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TITLE: Structure Optimization of 21,23-Core-Modified Porphyrins Absorbing Long-Wavelength Light as Potential Photosensitizers Against Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Youngjae You, Ph.D.

CONTRACTING ORGANIZATION: State University of New York  
Albany, New York 12201-0009

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14. ABSTRACT  In the first year, we made eighteen new 21,23-core modified porphyrins, determined their photophysical properties, and evaluated the biological properties of them. In the second year, the research had been focused on 1) analyzing quantitative structure-activity relationships (QSAR) of the porphyrins prepared in the first year using mathematical models, 2) measuring specific binding to peripheral benzodiazepine receptors (PBR), and 3) establishing new synthetic methods for novel structures and preparing them. From QSAR analysis, we found that the theoretical lipophilicity (MiLog P) was the major factor for their phototoxicity, in general; the molecular size was important at least in the core-modified porphyrins having two carboxylic acids. Unfortunately, we could not detect any specific binding of the most potent compound toward PBR. In the synthetic part, we successfully prepared eleven compounds of novel structures. The evaluation of them photophysically and biologically will be the major subjects of the third year in the project.					
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## Introduction

Photodynamic therapy (PDT) is a promising new treatment for cancer that is expected to be more selective and less toxic compared to current major treatment regimes such as surgery, chemotherapy and radiotherapy.<sup>1</sup> However, there are only a few photosensitizers approved for PDT, and they have properties far from an ideal photosensitizer.<sup>2</sup> The overall goal of this project is to obtain novel photosensitizers from 21,23-core-modified porphyrins targeting mitochondrial peripheral benzodiazepine receptor (PBR) for the PDT treatment of breast cancers.

21,23-Core-modified porphyrins are an attractive chemical entity as a lead compound with several merits: they absorb longer wavelengths of light ( $\geq 695\text{nm}$ ) and have established synthetic procedures that permit diverse structural modification.<sup>3-5</sup> PBR has been aimed as a primary target of various photosensitizers.<sup>6-8</sup> More interestingly, previous studies have shown that most breast cancer cell lines produce the PBR and, in several more aggressive breast cancer cell lines, the PBR is over-expressed.<sup>9-11</sup> Thus, we hypothesized that the design of core-modified porphyrins structurally similar to protoporphyrin IX, which has been known as natural ligand of PBR, might lead to more efficient photosensitizers for the treatment of breast cancers.

In the first year, we accomplished the following; 1) synthesis of eighteen new 21,23-core modified porphyrins, 2) determination of photophysical properties of these compounds, 3) measurement of phototoxicity of the compounds; and, 4) detection of apoptosis after the PDT treatment with the most potent derivative, compound **2**.<sup>5</sup>

## Body

In the second year, the research had been focused on 1) quantitative structure-activity relationships (QSAR) of porphyrins, 2) measurement of binding of PBR, 3) establishment of synthetic methods for novel structure of core-modified porphyrins, and 4) establishment of new synthetic methods for novel structures and preparation of them based on the methods.

### *QSAR of porphyrins in phototoxicity*

QSAR was performed to analyze the effects of structural properties of core-modified porphyrins on phototoxicity ( $\text{EC}_{50}$ ). The structural properties, so called molecular descriptors, of core-modified porphyrins were calculated by the computational methods using Sybyl or modules in the website of Molinspiration. All the calculations were carried out as all ionized form for the sulfonates and both ionized and unionized forms for carboxylic acids. The correlations between calculated descriptors and measured phototoxicity were determined and expressed in the mathematical models as either simple linear equations or three-dimensional models. From this QSAR analysis, we could tell the important molecular features of core-modified porphyrins for good phototoxicity and use this model in designing new structures.

To achieve a more through analysis of the relationships of structure of core-modified porphyrins with phototoxicity, we included the core-modified porphyrins previously prepared in our laboratory (Fig.1). In general, the calculated lipophilicity of the compounds was the most important molecular property in phototoxicity.  $\text{MiLogP}$ , calculated  $\log P$ , showed the strongest correlation with phototoxicity among the descriptors including MW (molecular weight), VOL (molecular volume), AREA (molecular area), and PSA (polar surface area).  $\text{MiLogP}$  seems to be even more important than physicochemical properties such as  $\text{ExCoff}$  (excitation coefficient), QYO (quantum yield of singlet oxygen), and  $\text{LogD}_{7.4}$ , which were experimentally measured. The relationships are expressed as follow and shown in Figure 1a.

$$\text{Log}(1/\text{EC}_{50}) = 0.31 \cdot \text{MiLogP} + 3.04, R_2 = 0.71 \text{ (- cp 22) when COOH (Fig. 1a)}$$

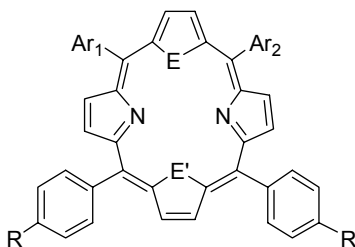
$$\text{Log}(1/\text{EC}_{50}) = 0.36 \cdot \text{MiLogP} + 2.83, R_2 = 0.58 \text{ (- cp 22) when COONa}$$

The compounds with higher  $\text{MiLogP}$  are more potent in phototoxicity, which is consistent with our previous qualitative SAR analyses:  $\text{EC}_{50}$ :  $4 \times \text{SO}_3\text{Na} < 2 \times \text{SO}_3\text{Na} < 2\text{COOH}$  and  $4 \times \text{COOH} \sim 3 \times \text{COOH} < 2 \times \text{COOH}$ .<sup>5, 12</sup>

However, there is a group of compounds which shows a different correlation from the whole set of the compounds in red circle in Figure 1a. These compounds are core-modified porphyrins having two carboxylic acids at one side of the molecular structures (compounds 1~20). To examine the QSAR in detail among these compounds, the relationships of steric and electronic descriptors with phototoxicity were analyzed. Of the molecular descriptors, one of the steric parameters, MA (molecular area), demonstrated a very strong correlation with phototoxicity (Fig. 1b). On the other hand, an electronic

parameter, PC (point charge at p-carbon at meso phenyl rings) did not show any correlation with phototoxicity.

Table 1. Data for the QSAR analysis: structures, Phototoxicity, and MiLog P of the core-modified porphyrins



Compd	E1, E2	Ar <sub>1</sub>	Ar <sub>2</sub>	R	EC <sub>50</sub> (M)	MA (Å <sup>2</sup> )	MiLog P
1	S, S	Phenyl (Ph)	Ph	OCH <sub>2</sub> COOH	1.5E-07	1258.9	10.805
2	S, S	2,4,5-tri-CH <sub>3</sub> -Ph	Ph	OCH <sub>2</sub> COOH	9.0E-08	1367.6	11.235
3	S, S	4-CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub> -Ph	4-CH <sub>3</sub> (CH <sub>2</sub> ) <sub>3</sub> -Ph	OCH <sub>2</sub> COOH	1.0E-06	1524.9	11.786
4	S, S	4-C <sub>6</sub> H <sub>6</sub> -Ph	4-C <sub>6</sub> H <sub>6</sub> -Ph	OCH <sub>2</sub> COOH	1.0E-06	1550.7	11.762
5	S, S	4-F-Ph	4-F-Ph	OCH <sub>2</sub> COOH	2.7E-07	1271.9	10.941
6	S, S	4-F-Ph	Ph	OCH <sub>2</sub> COOH	8.0E-08	1257.6	10.875
7	S, S	4-Cl-Ph	4-Cl-Ph	OCH <sub>2</sub> COOH	5.5E-07	1295.5	11.279
8	S, S	4-CF <sub>3</sub> -Ph	4-CF <sub>3</sub> -Ph	OCH <sub>2</sub> COOH	3.3E-07	1366.1	11.393
9	S, S	4-CF <sub>3</sub> -Ph	Ph	OCH <sub>2</sub> COOH	2.6E-07	1309.9	11.142
10	S, S	4-CH <sub>3</sub> O-Ph	4-CH <sub>3</sub> O-Ph	OCH <sub>2</sub> COOH	5.6E-07	1350.1	10.854
11	S, S	4-(CH <sub>3</sub> ) <sub>2</sub> N-Ph	4-(CH <sub>3</sub> ) <sub>2</sub> N-Ph	OCH <sub>2</sub> COOH	2.4E-07	1430.9	10.892
12	S, S	thiophenyl	thiophenyl	OCH <sub>2</sub> COOH	1.1E-07	1245.8	10.59
13	S, S	4-OH-Ph	4-OH-Ph	OCH <sub>2</sub> COOH	2.0E-06	1275.5	10.241
14	S, S	thiophenyl	Ph	OCH <sub>2</sub> COOH	8.0E-08	1251.8	10.703
15	S, S	4-CH <sub>3</sub> -Ph	4-CH <sub>3</sub> -Ph	OCH <sub>2</sub> COOH	1.3E-07	1333.9	11.142
16	S, S	4-CH <sub>3</sub> CH <sub>2</sub> -Ph	4-CH <sub>3</sub> CH <sub>2</sub> -Ph	OCH <sub>2</sub> COOH	6.5E-07	1403.6	11.403
17	S, S	4-(CH <sub>3</sub> ) <sub>2</sub> CH-Ph	4-(CH <sub>3</sub> ) <sub>2</sub> CH-Ph	OCH <sub>2</sub> COOH	1.0E-06	1445.4	11.66
18	S, S	4-(CH <sub>3</sub> ) <sub>3</sub> C-Ph	4-(CH <sub>3</sub> ) <sub>3</sub> C-Ph	OCH <sub>2</sub> COOH	8.6E-07	1477.3	11.731
19	S, S	4-(CH <sub>3</sub> ) <sub>3</sub> C-Ph	Ph	OCH <sub>2</sub> COOH	3.0E-07	1360.6	11.372
20	S, S	2,4,5-tri-CH <sub>3</sub> -Ph	2,4,5-tri-CH <sub>3</sub> -Ph	OCH <sub>2</sub> COOH	3.4E-07	1360.8	11.535
21	NH, NH	4-SO <sub>3</sub> Na-Ph	4-SO <sub>3</sub> Na-Ph	SO <sub>3</sub> Na	7.2E-05	-	4.481
22	S, S	4-CF <sub>3</sub> -Ph	4-CF <sub>3</sub> -Ph	SO <sub>3</sub> Na	1.0E-04	1289	8.16
23	S, S	4-(CH <sub>3</sub> ) <sub>2</sub> N-Ph	4-(CH <sub>3</sub> ) <sub>2</sub> N-Ph	SO <sub>3</sub> Na	6.4E-07	1345.7	8.573
24	S, S	1-SO <sub>3</sub> Na-4-CH <sub>3</sub> -Ph	1-SO <sub>3</sub> Na-4-CH <sub>3</sub> -Ph	SO <sub>3</sub> Na	1.2E-05	1287.3	6.723
25	S, S	4-SO <sub>3</sub> Na-Ph	4-SO <sub>3</sub> Na-Ph	SO <sub>3</sub> Na	3.0E-05	1303.3	5.97
26	S, NH	4-SO <sub>3</sub> Na-Ph	4-SO <sub>3</sub> Na-Ph	SO <sub>3</sub> Na	1.0E-04	1301.8	5.225
27	S, S	4-F-Ph	4-F-Ph	SO <sub>3</sub> Na	1.6E-06	1184.7	8.696

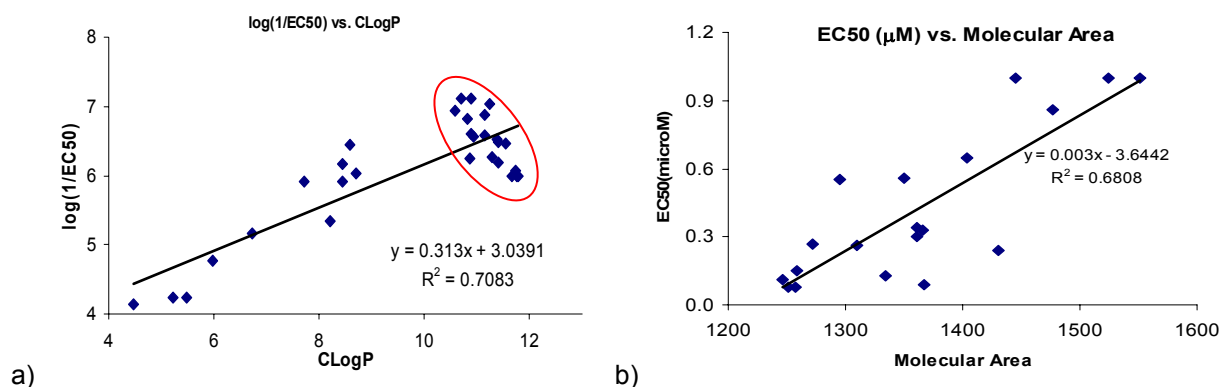


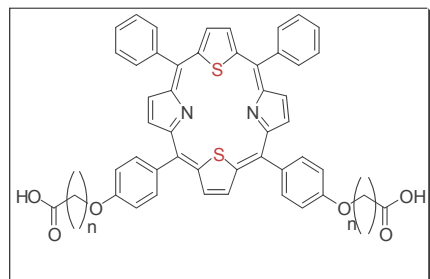
Figure 1. Correlations between a)  $\log(1/EC_{50})$  and MiLog P from compound **1** - **27**, b)  $EC_{50}$  and Molecular Area for compounds **1** - **20**.

### Binding of core-modified porphyrins to PBR

Different from our hypothesis, the most potent core-modified porphyrin, compound **2**, did not seem to specifically bind to PBR in mitochondria. We could not detect any specific binding of compound **2** to PBR in [<sup>3</sup>H] PK11195 binding studies in live cells using R3230AC cells. In addition, the image of cellular localization of compound **2** did not show specific localization in mitochondria. We are planning to explore sub-cellular localization of compound **2**.

### Synthetic methods for novel structures and preparation of new compounds

The modification of new compounds was focused on the unexplored part of core-modified porphyrins. The first set of compounds has different chain lengths between *meso*-phenyl and carboxylic acid groups. The compounds have 1, 3, 4, 5, 6, 10 methylenes, respectively. Interestingly, phototoxicity of the compounds was reversely dependent on the chain length: the shorter of the chain length, the more potent in phototoxicity. It is hypothesized that if the chain length is getting longer, the lipophilicity of the compounds may become too high. Thus, the core-modified porphyrins form more tight aggregates which are unfavorable for biological activity.



compd	no. of CH <sub>2</sub>	EC <sub>50</sub> (μM)	$\lambda_{max}$ (nm)	$\epsilon$ (x 10 <sup>3</sup> M <sup>-1</sup> cm <sup>-1</sup> )
<b>1</b>	1	0.15	435	314
<b>28</b>	3	0.14	438	224
<b>29</b>	4	0.42	438	157
<b>30</b>	5	1.63	438	110
<b>31</b>	6	> 10	438	91
<b>32</b>	10	> 10	438	176

Table 2. Structures of new core-modified porphyrins with various chain length and phototoxicity of them.

The next set of compounds have new structural features compared to the compounds prepared in the first year: 1) core-modified porphyrins with mono functional groups having either carboxylic acid or sulfonate group, 2) chlorine-type dithiaporphyrins with diols in the porphyrin core, and 3) core-modified porphyrins without two *meso*-aryl groups. The core-modified porphyrins with mono-functional group were synthesized based on our previous method.<sup>13</sup> The chlorine-type dithiaporphyrins were made by oxidation with OsO<sub>4</sub>, which gave compound of *syn*-diols.<sup>14</sup> To make the core-modified porphyrins without two *meso*-aryl groups, new diols compound was prepared through a novel intermediate. (to be published shortly) The photophysical property and biological activity of these compounds will be determined in the third year.

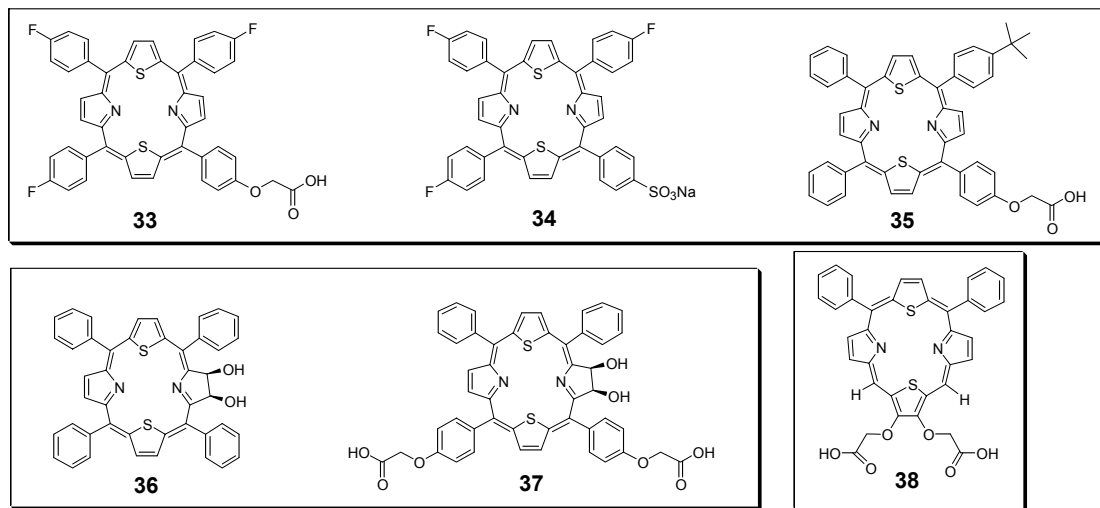


Figure 3. Structures of novel compounds prepared during the second year

### Key Research Accomplishments

**Training:** The PI acquired the statistical knowledge and the computational techniques necessary to perform QSAR analysis. The PI obtained a tenured-track faculty position in the Department of Chemistry and Biochemistry at the South Dakota State University.

**Research accomplishments:** 1) The QSAR models of the core-modified porphyrins related to phototoxicity were built. 2) Synthesis of novel core-modified porphyrins was prepared and characterized.

### Reportable Outcomes

#### Publications

1. The part of the report was already published in peer-reviewed journal: Youngjae You, Scott L. Gibson and Michael R. Detty, Core-modified porphyrins. Part 5: Electronic effects on photophysical and biological properties in vitro. *Bioorganic & Medicinal Chemistry*, 13 (6), 5968-5980, 2005
2. The other part of the first year results is submitted to *Journal of Photochemistry and Photobiology – Biology B: Biology*: Youngjae You, Scott L. Gibson and Michael R. Detty Phototoxicity of a core-modified porphyrin and induction of apoptosis

#### Posters

1. The 229th American Chemical Society National Meeting, Mar 13-17, 2005, San Diego, CA, You, Y.; Gibson S. L.; Hilf R.; Detty, M. R. SAR study of new core-modified porphyrins as photosensitizers for photodynamic cancer therapy (*This abstract was missed last annual report.*)
2. The 4th Era of Hope meeting for the Department of Defense (DOD) Breast Cancer Research Program (BCRP), Jun 8-11, 2005, Philadelphia, PA, You, Y.; Gibson, S. L.; Hilf R.; Detty, M. R. Structure-Activity Relationship of 21,23-Core-Modified Porphyrins for Photodynamic Therapy of Cancer
3. The 230th American Chemical Society National Meeting, Aug 28-Sep 1, 2005, Washington, DC, You, Y.; Gibson S. L.; Hilf R.; Detty, M. R. SAR of new core-modified porphyrins as photosensitizers for photodynamic cancer therapy: electronic effects and importance of aggregation in biological activity

#### Invited Seminars for an Interview

1. Department of Chemistry, Oklahoma State University, Stillwater, OK, Dec. 12, 2005.
2. Department of Chemistry and Biochemistry, Montclair State University, Montclair, NJ, Dec. 16, 2005.
3. Arnold & Marie Schwartz College of Pharmacy and Health sciences, Long Island University, Brooklyn, NY, Dec. 20, 2005.
4. Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, GA, Jan. 26, 2006.
5. Department of Chemistry and Biochemistry, South Dakota State University, Feb. 21, 2006.
6. Department of Medicinal Chemistry and Pharmacognosy, University of Chicago, Chicago, IL, Feb. 17, 2006.
7. Department of Medicinal & Biological Chemistry, University of Toledo, Feb. 29.

### Conclusions

The progress in the synthetic part of research is satisfactory based on the proposed SOW. However, more concentrated efforts in the biological part is necessary, especially for *in vivo* study. The PI and new subcontractor are planning to do *in vivo* study shortly. After the second year, the PI is qualified to perform QSAR analysis with the aid of computational methods, especially in case of ligand-based approach. More encouraging outcome was achieved in terms of training for new independent scientists: the PI safely landed an academic position where he can continue his current research of photodynamic therapy for breast cancers.

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## Appendices

1. The journal article: Bioorganic & Medicinal Chemistry, 13 (6), 5968-5980, 2005
2. The manuscript submitted to Journal of photochemistry and photobiology B: Biology
3. The abstract for the 229th American Chemical Society National Meeting
4. The abstract for the 230th American Chemical Society National Meeting
5. The abstract for the 4th Era of Hope meeting for the Department of Defense Breast Cancer Research Program



## Core-modified porphyrins. Part 5: Electronic effects on photophysical and biological properties in vitro

Youngjae You,<sup>a,\*</sup> Scott L. Gibson<sup>b</sup> and Michael R. Detty<sup>a</sup>

<sup>a</sup>*Institute for Lasers, Photonics, and Biophotonics, Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, NY 14260, USA*

<sup>b</sup>*Department of Biochemistry and Biophysics, University of Rochester Medical Center, 601 Elmwood Avenue, Box 607, Rochester, NY 14642, USA*

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**Abstract**—21,23-Dithiaporphyrins **2–11** were prepared as analogues of 5,20-diphenyl-10,15-bis(4-carboxylatomethoxy)phenyl-21,23-dithiaporphyrin **1** to examine the impact of electronic properties at the 5- and 20-*meso*-positions. The effects of the electronic properties at the *meso*-rings were not significant with respect to absorption spectra, quantum yields for the generation of singlet oxygen and for fluorescence. While some differences were noted in the *n*-octanol/pH 7.4 buffer partition coefficient,  $\log D_{7.4}$ , among the compounds,  $\log D_{7.4}$  did not critically influence the cellular uptake or phototoxicity. None of the dithiaporphyrins **1–11** displayed dark toxicity at concentrations up to  $1 \times 10^{-5}$  M. Once irradiated with  $5 \text{ J cm}^{-2}$  of 350–750 nm light, five porphyrins **2**, **3**, **5**, **6**, and **8** killed over 80% of R3230AC rat mammary adenocarcinoma cells at  $5 \times 10^{-7}$  M photosensitizer. Among these five, compound **3** bearing 5-phenyl and 20-(4-fluorophenyl) substituents was the most potent photosensitizer toward R3230AC cells showing 67% cell kill at  $1 \times 10^{-7}$  M **3**. Bulky substituents at the 5- and 20-positions gave photosensitizers with minimal phototoxicity.

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### 1. Introduction

Photodynamic therapy (PDT) is a promising new treatment regimen for cancer that uses three components to destroy the tumor: a photosensitizer, light, and oxygen.<sup>1–9</sup> The photosensitizer generates cytotoxic singlet oxygen following absorption of a photon of appropriate wavelength. Photofrin has gained regulatory approval in many countries but it has several drawbacks, such as long-term skin phototoxicity, chemical complexity, and weak absorption at shorter wavelengths (~630 nm).<sup>10</sup> Therefore, the development of more “ideal” photosensitizers is a major emphasis in research on photodynamic therapy, which should be chemically pure, generate singlet oxygen efficiently, accumulate selectively in the tumor, and observe longer wavelength light (650–800 nm). Currently, several new photosensitizers are approved or are under clinical study.<sup>11</sup>

Dithiaporphyrins have been extensively studied in our laboratory as second-generation photosensitizers due to several advantages. The various dithiaporphyrins can be prepared in high purity through established synthetic schemes, can absorb longer wavelengths of light (690–710 nm) than natural tetra nitrogenic porphyrins (~630 nm), and are more photostable than Photofrin upon irradiation.<sup>12</sup> Dithiaporphyrins with two carboxylic acids exhibited a higher phototoxic activity toward R3230AC rat mammary adenocarcinoma cells than derivatives with one, three, or four acid groups.<sup>13</sup> Bulky substituents at the *meso*-positions reduced phototoxicity, while effects on physicochemical properties, such as absorption spectra and quantum yields for the generation of singlet oxygen and fluorescence, were minimal.<sup>14</sup> Of the dithiaporphyrins prepared in our previous study, 5-phenyl-20-(2-thienyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (**IY69**) showed the most potent phototoxic activity and irradiation of cells treated with **IY69** induced apoptotic cell death along with the damage to mitochondrial function.<sup>14</sup>

The phototoxicity of **IY69** prompted us to develop a more extensive study of substituent effects in the dithiaporphyrin diacids. The structures of compounds in this

**Keywords:** Core-modified porphyrin; Dithiaporphyrin; Photodynamic therapy; Anticancer therapy; QSAR.

\* Corresponding author. Tel.: +1 716 645 6800x2197; fax: +1 716 645 6963; e-mail: [yjyou@nsm.buffalo.edu](mailto:yjyou@nsm.buffalo.edu)

work were designed to investigate the electronic effects of substituents at the *para*-positions of *meso*-phenyl rings on physicochemical and biological outcomes. Cellular uptake and photo- and dark toxicity of the various dithiaporphyrins were evaluated along with the important physicochemical properties for a photosensitizer, such as absorption spectra, quantum yields for the generation of singlet oxygen and for fluorescence, and the *n*-octanol/water partition coefficient at pH 7.4, log  $D_{7.4}$ .

## 2. Results and discussion

### 2.1. Chemistry

**2.1.1. Synthesis of symmetrical 21,23-dithiaporphyrins 4, 5, and 7–11.** The synthesis of symmetric core-modified porphyrins **4**, **5**, **8**, **10**, and **11** (Fig. 1) followed our previous method.<sup>12–14</sup> As shown in Scheme 1, 2,5-bis[1-(4-substituted-phenyl)-1-hydroxymethyl]thiophene diols **12**, **13**, and **15–17** were synthesized from thiophene and the corresponding aldehydes in 70–75% isolated yields. The diols were condensed with 2,5-bis[1-(4-methoxyphenyl)-1-pyrrolomethyl]-thiophene **22** and oxidized

in the presence of 2,3,5,6-tetrachlorobenzoquinone (TCBQ) and *p*-toluenesulfonic acid monohydrate (TsOH·H<sub>2</sub>O) or boron trifluoride etherate (BF<sub>3</sub>·OEt<sub>2</sub>) in CH<sub>2</sub>Cl<sub>2</sub> giving dimethoxy dithiaporphyrins **24–28** in 3–18% isolated yields (Scheme 2). Demethylation with boron tribromide (BBr<sub>3</sub>) in CH<sub>2</sub>Cl<sub>2</sub> gave diphenolic dithiaporphyrins **31–35** (83–95%), which were then alkylated in the presence of K<sub>2</sub>CO<sub>3</sub> and ethyl bromoacetate in acetone to produce ester porphyrins **36–40**. Final diacid dithiaporphyrins **4**, **5**, **8**, **10**, and **11** were prepared by hydrolysis of the esters with NaOH in a 1:1 solution of distilled water and tetrahydrofuran (THF).

In the preparation of di-4-methoxyphenyl- and di-4-hydroxyphenyldithiaporphyrin **7** and **9**, the upper scheme could not be applied due to possible problems in either the demethylation of **7** or the alkylation of **9**. Consequently, slightly modified routes were devised (Scheme 2). To avoid any difficulty in selective demethylation of a tetramethoxy porphyrin in the preparation of dimethoxy porphyrin **7**, ester porphyrin **29** was prepared directly by cyclization of 2,5-bis[1-(4-methoxyphenyl)-1-

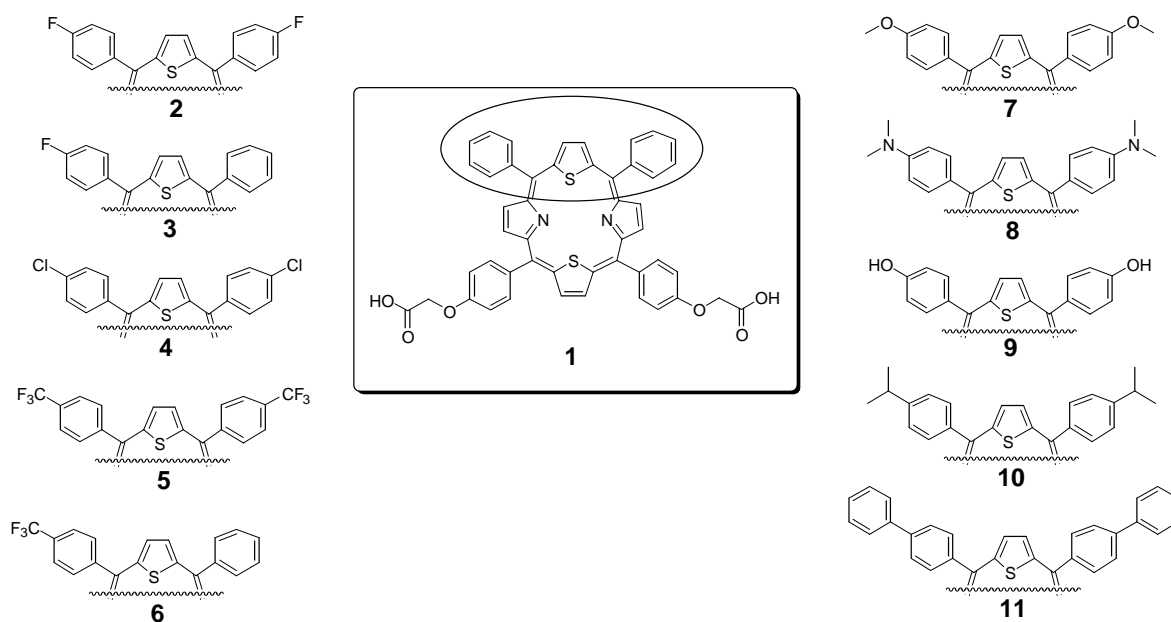
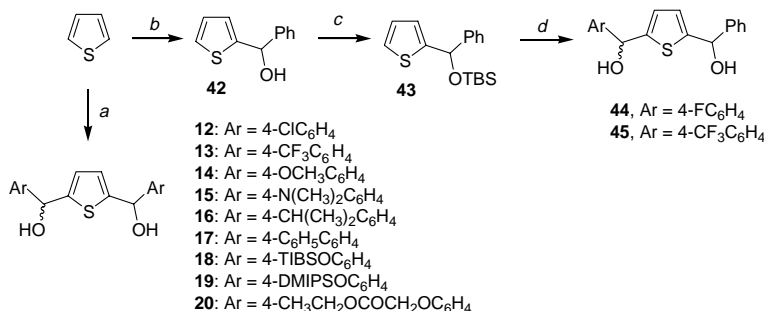
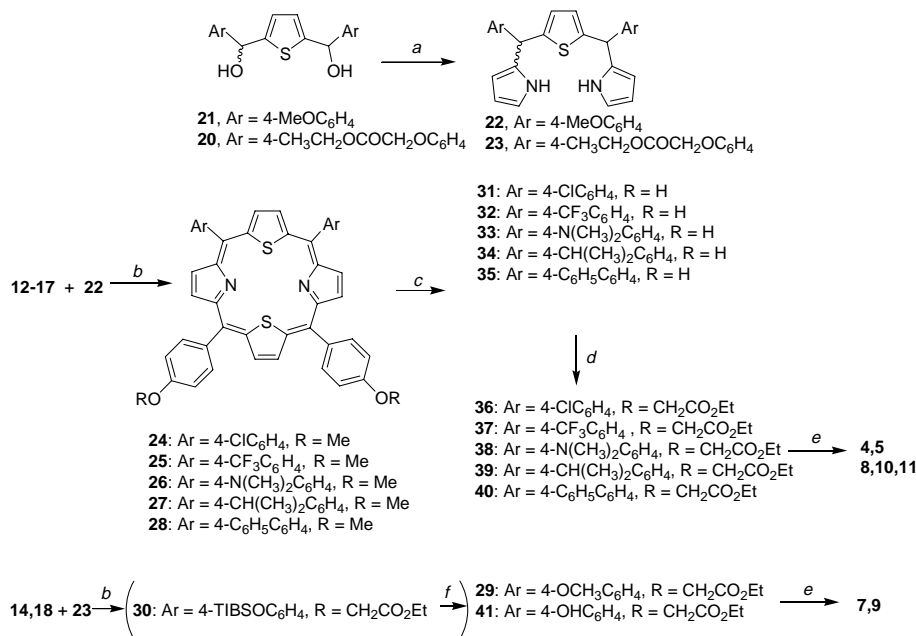


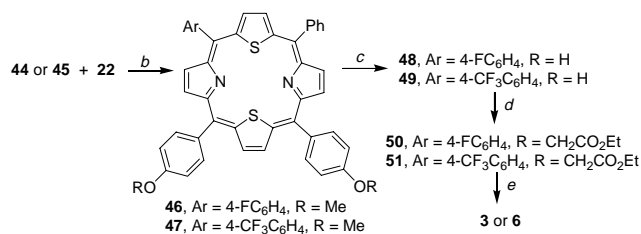
Figure 1. Structures of 21,23-dithiaporphyrins with various group substitutions at the 5,20-*meso*-positions.



Scheme 1. Reagents: (a) i. 2.5 equiv *n*-BuLi, ii. 2 equiv aryl aldehyde; (b) i. 1 equiv BuLi, ii. 1 equiv benzaldehyde; (c) TBSCl, DMAP, Et<sub>3</sub>N; (d) i. 1 equiv *n*-BuLi, ii. 1 equiv aryl aldehyde, iii. aqueous HCl.



**Scheme 2.** Reagents: (a) pyrrole, BF<sub>3</sub>·OEt<sub>2</sub>; (b) TCBQ, TsOH, CH<sub>2</sub>Cl<sub>2</sub>; (c) BBr<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>; (d) BrCH<sub>2</sub>CO<sub>2</sub>Et, K<sub>2</sub>CO<sub>3</sub>, acetone; (e) NaOH, aqueous THF; (f) TBAF.



**Scheme 3.** Reagents: (a) pyrrole, BF<sub>3</sub>·OEt<sub>2</sub>; (b) TCBQ, TsOH, CH<sub>2</sub>Cl<sub>2</sub>; (c) BBr<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>; (d) BrCH<sub>2</sub>CO<sub>2</sub>Et, K<sub>2</sub>CO<sub>3</sub>, acetone; (e) NaOH, aqueous THF.

hydroxymethyl]thiophene **14** and diethyl 2,5-bis[1-(4-carboxylatomethoxyphenyl)-1-pyrrolomethyl]thiophene **23**. For the preparation of **9**, protection of the phenolic functionality was necessary. Protected diol, 2,5-bis[1-(4-triisobutylsiloxy)phenyl]-1-hydroxymethyl]thiophene **18**, was synthesized and condensed with **23** to give hydroxyl-protected ester **30** in 32% isolated yield. Deprotection of **30** with tetrabutylammonium fluoride (TBAF) in THF provided ester **41**.

**2.1.2. Synthesis of unsymmetrical dicarboxylic acid derivatives 3 and 6.** Reported methods to prepare unsymmetrical core-modified porphyrins were used in the preparation of dithiaporphyrins **3** and **6** with 4-fluorophenyl and 4-trifluoromethylphenyl substituents, respectively (Scheme 3). Unsymmetric diols **44** and **45** were prepared from 2-lithiothiophene via the *tert*-butylsilyl (TBS)-protected monohydroxythiophene **43**. Cyclization of **44** and **45** with **22** gave dimethoxydithiaporphyrins **46** and **47** in 5% and 7% isolated yields, respectively. Diacid porphyrins **3** and **6** were successfully synthesized through demethylation, alkylation, and hydrolysis, under the same conditions as those used for the symmetrical analogues.

## 2.2. Photophysical properties

**2.2.1. Absorption maxima.** The absorption maximum and molar extinction coefficient of band I of porphyrins are important parameters in evaluating potential photosensitizers for PDT. Longer wavelength light (650–800 nm) reaches deeper into tissue to activate photosensitizers.<sup>15</sup> Photosensitizers with higher extinction coefficients harvest the irradiating light more efficiently. So, photosensitizers having longer-wavelength absorption maxima (~800 nm) and higher extinction coefficient are highly desirable. Although most 4-aryl substituents at the *meso*-positions have minimal impact on the absorption maximum of band I, introduction of fluoro **2** or dimethylamino **8** groups results in a significant redshift from the diphenyl derivative **1** ( $\Delta\lambda_{\max} = +19$  and  $+16$  nm, respectively, Table 1). Also noteworthy is the extinction coefficient of band I for compound

**Table 1.** UV–vis–near-IR band maxima and molar absorptivities for dithiaporphyrins **1–11** in methanol<sup>a</sup>

Compound	Soret	Band IV	Band III	Band II	Band I
<b>1</b>	435 (314)	513 (27.7)	549 (10.7)	632 (3.2)	698 (7.0)
<b>2<sup>b</sup></b>	442 (127)	533 (14.0)	573 (10.8)	645 (2.5)	717 (4.8)
<b>3</b>	435 (407)	514 (26.9)	548 (9.9)	634 (1.8)	698 (5.3)
<b>4</b>	436 (296)	515 (21.4)	550 (8.8)	634 (1.7)	699 (4.6)
<b>5</b>	435 (297)	514 (24.3)	548 (9.2)	633 (1.8)	697 (4.6)
<b>6</b>	435 (327)	514 (25.4)	549 (11.3)	634 (2.1)	698 (5.6)
<b>7<sup>c</sup></b>	440 (176)	517 (14.6)	554 (10.3)	638 (1.2)	704 (4.1)
<b>8<sup>c</sup></b>	449 (100)	522 (17.7)	573 (19.4)	—	714 (8.9)
<b>9<sup>c</sup></b>	440 (263)	518 (21.5)	555 (12.3)	639 (1.6)	704 (5.9)
<b>10</b>	436 (306)	515 (21.6)	551 (9.6)	635 (1.6)	700 (5.5)
<b>11<sup>c</sup></b>	439 (97.6)	518 (13.7)	553 (7.5)	637 (1.1)	702 (3.8)

<sup>a</sup>  $\lambda_{\max}$  nm ( $\epsilon \times 10^3$  M<sup>-1</sup> cm<sup>-1</sup>).

<sup>b</sup> Di-sodium salts and data from Ref. 12.

<sup>c</sup> In THF.

**8** ( $8.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ), which is the highest among the dithiaporphyrin derivatives.

**2.2.2. Quantum yields for the generation of singlet oxygen.** Singlet oxygen ( $^1\text{O}_2$ ) is thought to be the toxic species that damages tumor cells following irradiation of the photosensitizer.<sup>16</sup> Higher quantum yields for the generation of singlet oxygen [ $\phi(^1\text{O}_2)$ ] endow photosensitizers with greater potential for biological activity, that is, phototoxicity. Values of  $\phi(^1\text{O}_2)$  for **1–11** were determined by direct detection of singlet-oxygen luminescence at 1270 nm, which was compared with the standard, rose Bengal [ $\phi(^1\text{O}_2) = 0.80$ ]. Excitation was effected with a frequency-doubled neodymium–YAG laser emitting at 532 nm. All the core-modified porphyrins **1–11** showed high values of  $\phi(^1\text{O}_2)$  ( $>0.73$ ), with the exception of two compounds, 4-dimethylaminophenyl derivative **8** [ $\phi(^1\text{O}_2) = 0.32$ ] and 4-hydroxyphenyl derivative **9** [ $\phi(^1\text{O}_2) = 0.03$ ] (Table 2).

**2.2.3. Quantum yields for fluorescence.** Molecular fluorescence is a useful tool to study the localization and pharmacokinetics of dyes both in vitro and in vivo.

Quantum yields of fluorescence ( $\phi_F$ ) were determined for the compounds **1–11** compared to rhodamine 6G ( $\phi_F = 1.0$ ). Interesting enough is the increase of  $\phi_F$  of compound **8** (0.029), which is theoretically consistent with its low value of  $\phi(^1\text{O}_2)$ , 0.32 (Table 2). In contrast, compound **9** displayed low quantum yields for both the generation of singlet oxygen [ $\phi(^1\text{O}_2) = 0.03$ ] and for fluorescence ( $\phi_F = 0.002$ ). All the other compounds have poor fluorescence with values of  $\phi_F < 0.012$ .

**2.2.4. *n*-Octanol/water partition coefficients.** The lipophilicity of a molecule is an important determinant of biomembrane permeability. The partition between *n*-octanol and pH 7.4 buffer was measured by a slightly modified ‘shake-flask’ method.<sup>17</sup> The change in substituents on the *meso*-aryl groups gave a range of values of  $\log D_{7.4}$  from  $-0.16$  for 4-isopropylphenyl derivative **10** to  $0.78$  for 4-hydroxyphenyl derivative **9** (Table 2). Interestingly, the effects of the hydroxyl and isopropyl groups were contrary to our expectations. The isopropyl group increases  $\log D_{7.4}$  ( $\Delta \log D_{7.4} = +0.74$ ) and hydroxyl group decreases  $\log D_{7.4}$  ( $\Delta \log D_{7.4} = -0.20$ ) from compound **1** ( $\log D_{7.4} = 0.04$ , Table 2), even though the hydrophobic constants ( $\pi$ ) of isopropyl and hydroxyl groups are  $1.53$  and  $-0.67$ , respectively.<sup>18</sup> This observation strongly suggests that  $\log D_{7.4}$  might be influenced by other factors, such as dimerization or aggregation in solution.

**Table 2.** Quantum yields for the generation of singlet oxygen [ $\phi(^1\text{O}_2)$ ], quantum yields for fluorescence ( $\phi_F$ ), and *n*-octanol/water partition coefficients in pH 7.4 phosphate buffer ( $\log D_{7.4}$ ) for **1–11**<sup>a</sup>

Compound	$\phi(^1\text{O}_2)$	$\phi_F$	$\log D_{7.4}$
<b>1</b>	0.80	0.007	$0.04 \pm 0.02$
<b>2</b>	0.71 <sup>b</sup>	0.003 <sup>b</sup>	ND <sup>c</sup>
<b>3</b>	0.83	0.010	$-0.06 \pm 0.01$
<b>4</b>	0.76	0.011	$0.19 \pm 0.06$
<b>5</b>	0.75	0.010	$0.27 \pm 0.05$
<b>6</b>	0.87	0.009	$-0.08 \pm 0.01$
<b>7</b>	0.81	0.012	$-0.15 \pm 0.08$
<b>8</b>	0.32	0.029	$0.56 \pm 0.15$
<b>9</b>	0.03	0.002	$0.78 \pm 0.08$
<b>10</b>	0.74	0.010	$-0.16 \pm 0.06$
<b>11</b>	0.73	0.012	$0.12 \pm 0.14$

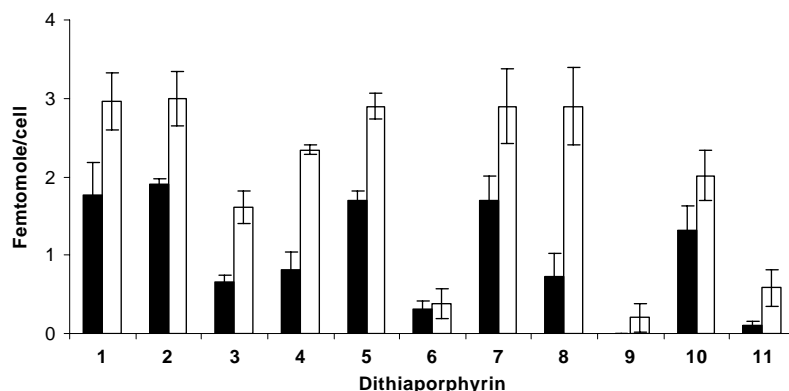
<sup>a</sup> Detailed methods for measurements above are presented in Section 4.

<sup>b</sup> Ref. 12.

<sup>c</sup> ND (not determined) indicates samples for which measurements were not performed.

### 2.3. Biology

**2.3.1. Intracellular accumulation of core-modified porphyrins into cultured R3230AC cells.** The cellular uptake of core-modified porphyrins after 24 h incubation was determined by fluorescence techniques (Fig. 2). Following exposure to photosensitizers, cells were digested and the porphyrins were solubilized with 25% Scintigest (100% DMSO for compound **9**). Owing to low values of  $\phi_F$  for porphyrins (Table 2), higher concentrations of photosensitizer were employed,  $5 \times 10^{-6}$  and  $1 \times 10^{-5}$  M, in the uptake experiment, relative to the phototoxicity experiments, although there was no significant dark toxicity with any of the compounds at  $1 \times 10^{-5}$  M.



**Figure 2.** Cellular uptake of 21,23-dithiaporphyrins in cultured R3230AC rat mammary adenocarcinoma cells. Each bar represents the mean intracellular uptake of each compound incubated with R3230AC cells for 24 h at  $5 \times 10^{-6}$  M (black bars) or  $1 \times 10^{-5}$  M (white bars). Data are expressed as femtomole porphyrin/cell and error bars are the SEM.

Cellular uptake of the dithiaporphyrins at  $5 \times 10^{-6}$  M of the photosensitizer covered a range from 0.1 fmol/cell for **11** to 1.9 fmol/cell for **2**. Cellular uptake was similar for the symmetrically substituted dithiaporphyrins **1**, **2**, **5**, **7**, and **10** (1.8, 1.9, 1.7, 1.7, and 1.8 fmol/cell, respectively) and was somewhat lower for 4-chlorophenyl derivative **4** (0.8 fmol/cell) and 4-dimethylaminophenyl derivative **8** (0.7 fmol/cell). Cellular uptake of the unsymmetrically substituted derivatives **3** (0.7 fmol/cell) and **6** (0.3 fmol/cell) was significantly less than the corresponding symmetrical derivatives **1**, **2**, and **5** for these substituents ( $P < 0.05$  for all pairwise comparisons). The lowest cellular uptake was observed with compound **11** (0.1 fmol/cell) with bulky biphenyl substituents. The value of  $\phi_F$  for **9** is much smaller than any of the other derivatives and no fluorescence could be detected following cell digestion of cells treated with  $5 \times 10^{-6}$  M photosensitizer. At  $1 \times 10^{-5}$  M photosensitizer, uptake of compound **9** (0.2 fmol/cell) was comparable to the uptake of compounds **6** (0.4 fmol/cell) and **11** (0.6 fmol/cell), but the uptake of these compounds was still less than for any of the others at either  $5 \times 10^{-6}$  M or  $1 \times 10^{-5}$  M.

The uptake does not appear to be strictly a function of lipophilicity.  $\log D_{7.4}$  values for compounds **1**, **5**, **7**, and **10** with the highest cellular uptake and compounds **6** and **11** with two of the lowest values of cellular uptake are all near 0. Additionally, compound **9** with the highest value of  $\log D_{7.4}$  at 0.78 showed the lowest uptake, which might be explained by either loss of amphiphilicity of the molecule or formation of aggregates in the media.

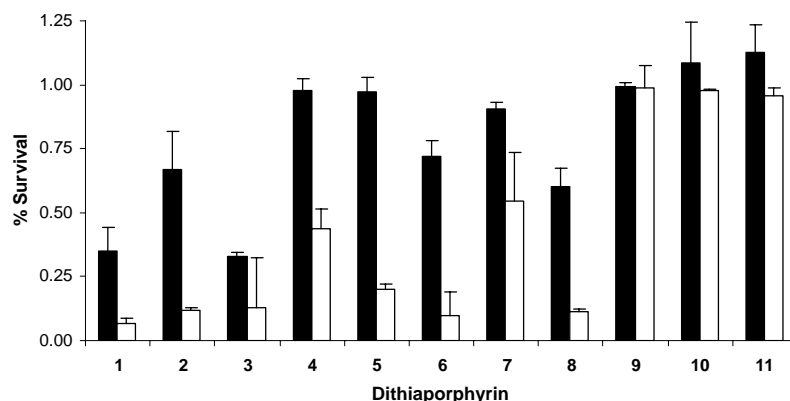
**2.3.2. Dark toxicity and phototoxicity of core-modified porphyrins toward cultured R3230AC cells.** Dark toxicity and phototoxicity of the core-modified porphyrins toward R3230AC rat mammary adenocarcinoma cells were determined using the MTT colorimetric assay. Cells were incubated for 24 h with  $5 \times 10^{-8}$  to  $1 \times 10^{-6}$  M core-modified porphyrin. Treated cells were then irradiated with  $1.4 \text{ mW cm}^{-2}$  broadband light (350–750 nm) for 1 h giving a total fluence of  $5 \text{ J cm}^{-2}$ . Cell survival was then determined 24 h following irradiation of treated cells and 24 h later for dark controls.

For the dark controls, cell survival was  $>85\%$  for all dithiaporphyrins at concentrations up to  $1 \times 10^{-5}$  M.

Phototoxicity with the porphyrins at  $1 \times 10^{-7}$  and  $5 \times 10^{-7}$  M is given in Figure 3. Dithiaporphyrins **2–6** with the electron-withdrawing substituents  $-\text{F}$  and  $-\text{CF}_3$ , dithiaporphyrin **8** with dimethylamino substituents, and dithiaporphyrin **1** with phenyl substituents showed comparable phototoxicity with  $<20\%$  survival at  $5 \times 10^{-7}$  M. However, compound **3** with *meso*-phenyl and 4-fluorophenyl substituents gave only 33% cell survival in R3230AC cells treated with  $1 \times 10^{-7}$  M **3**. Dithiaporphyrin **4** with chloro substituents and dithiaporphyrin **7** with methoxy substituents were not as phototoxic with treated cells displaying 44% and 54% cell survival, respectively, at  $5 \times 10^{-7}$  M. Dithiaporphyrin **9** with hydroxyl substituents and dithiaporphyrins **10** and **11** with bulky substituents showed essentially no phototoxicity at  $5 \times 10^{-7}$  M. Even at  $1 \times 10^{-6}$  M, compounds **10** and **11** showed 63% and 75% survival, respectively, with  $5 \text{ J cm}^{-2}$  of 350–750 nm light in treated cells. This is consistent with our earlier observations on dithiaporphyrins with two bulky substituents.<sup>14</sup>

Examination of the quantum yields for the generation of singlet oxygen, cellular uptake, and phototoxicity of the various dithiaporphyrins revealed some inconsistencies. Compounds **1**, **2**, **6**, and **8** were the most phototoxic compounds in the series **1–11**, yet  $\phi(^1\text{O}_2)$  was 0.80 for **1**, 0.71 for **2**, and 0.87 for **6** in solution but only 0.32 for **8**. Furthermore, cellular uptake was 1.8 fmol/cell for **1**, 1.9 fmol/cell for **2**, and only 0.3 and 0.7 fmol/cell for **6** and **8**, respectively, when the R3230AC cells were exposed to  $5 \times 10^{-6}$  M photosensitizer. In contrast, the photosensitizer **10** with  $\phi(^1\text{O}_2)$  of 0.73 and cellular uptake of 1.3 fmol/cell in cells treated with  $5 \times 10^{-7}$  M **10** was one of the poorest photosensitizers in the series. Clearly, efficacy of photosensitizers in the series **1–11** cannot be predicted by either values of  $\phi(^1\text{O}_2)$  or by cellular uptake or by a combination of the two.

Values of  $\log D_{7.4}$  also did not correlate with efficacy. Compounds **1**, **2**, **6**, and **10** had values of  $\log D_{7.4}$  near 0, while compound **8** was more lipophilic with  $\log D_{7.4}$  of 0.56. Compound **10** showed no phototoxicity in



**Figure 3.** Cell viability of cultured R3230AC cells after photosensitization with  $5 \text{ J cm}^{-2}$  of broadband light (350–750 nm) in the presence of 21,23-dithiaporphyrins,  $1 \times 10^{-7}$  M (black bars) or  $5 \times 10^{-7}$  M (white bars). Each data point represents the mean of at least three separate experiments performed in duplicate and error bars are the SEM. Data are expressed as the surviving fraction of viable cells relative to untreated controls.



marked contrast to compounds **1**, **2**, and **6** with similar values of  $\log D_{7.4}$ , uptake, and  $\phi(^1\text{O}_2)$ . Furthermore, the more lipophilic compound **8** was similar in potency to the amphiphilic molecules **1**, **2**, and **6**. In the absence of a single parameter to predict phototoxic behavior, one needs to consider the possibilities of differences in uptake and phototoxicity among monomeric and aggregate species, as well as the possibility of specific cellular sites of localization.

### 3. Summary and conclusions

As an extension of our previous study of structure–activity relationships that focused on the effects of steric bulk and symmetry in dithiaporphyrins, ten new core-modified porphyrins were prepared whose substituents varied in electronic properties. These derivatives were successfully prepared through the synthetic methods developed in our previous work.<sup>13</sup> However, the preparation of dimethoxy and dihydroxy compounds, **7** and **9**, respectively, required new synthetic approaches, as shown in Scheme 2.

The differences in electronic properties among the substituents had minimal impact on physical and photophysical features, such as absorption maxima ( $\lambda_{\text{max}}$ ), quantum yields for the generation of singlet oxygen [ $\phi(^1\text{O}_2)$ ] and fluorescence ( $\phi_{\text{F}}$ ), and *n*-octanol/water partition coefficients ( $\log D_{7.4}$ ). Compounds **2** and **8** with two 4-fluorophenyl or two 4-dimethylaminophenyl substituents in the *meso*-positions displayed a small bathochromic shift in  $\lambda_{\text{max}}$ , relative to the other derivatives, compound **8** had a value of  $\phi(^1\text{O}_2)$  that was less than half of any other derivative in the series, and compounds **8** and **9** were both more lipophilic than other derivatives in the series based on the values of  $\log D_{7.4}$ .

In the series **1–11**, five of the dithiaporphyrins (compounds **2**, **3**, **5**, **6**, and **8**) were efficient photosensitizers, giving less than 20% cell survival in R3230AC rat mammary adenocarcinoma cells following treatment with  $5 \times 10^{-7}$  M photosensitizer and  $5 \text{ J cm}^{-2}$  of 350–750 nm light. Compound **3** with *meso*-phenyl and 4-fluorophenyl substituents was the most potent photosensitizer among these five toward R3230AC cells, with only 33% cell survival following treatment with  $1 \times 10^{-7}$  M photosensitizer and  $5 \text{ J cm}^{-2}$  of 350–750 nm light. Dark controls indicated that there was no significant cellular toxicity in the absence of light.

Empirically, the substituent studies of this manuscript and our previous work have identified two dithiaporphyrins with two 4-(carboxylatomethoxy)phenyl substituents at the 10- and 15-positions, a phenyl substituent at the 5-position, and either a 4-fluorophenyl substituent (compound **3** of this study) or a 2-thienyl substituent (**IY69** of our previous study)<sup>14</sup> at the 20-position as superior photosensitizers with submicromolar  $\text{EC}_{50}$ 's and  $5 \text{ J cm}^{-2}$  of 350–750 nm light. As was observed in our earlier work with dithiaporphyrins with two 2,4,6-trimethylphenyl substituents or two 4-*tert*-butylphenyl substituents,<sup>14</sup> compounds **10** and **11** with two bulky

4-isopropylphenyl or 4-biphenyl substituents, respectively, showed essentially no phototoxicity. The presence of two bulky *meso*-substituents leads to an ineffective photosensitizer. In studies of over thirty dithiaporphyrin derivatives, no clear correlation of either physical or photophysical properties has allowed prediction of efficacy. While two derivatives appear to be superior in studies *in vitro*, we have as yet to determine a molecular target, although mitochondria are implicated as a general site of action.<sup>14</sup> The efficacy of **IY69** and compound **3** may be due to a specific molecular target or may be due to some physical phenomenon peculiar to these derivatives, relative to the others, such as aggregation/deaggregation in the cell. Future studies will focus on compounds **IY69** and **3** to identify reasons for their added efficacy.

## 4. Experimental

### 4.1. General methods

Solvents and reagents were used as received from Sigma–Aldrich Chemical (St. Louis, MO) unless otherwise noted. Cell culture medium was purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). Concentration *in vacuo* was performed on a Buchi rotary evaporator. NMR spectra were recorded at 23 °C on a Varian Gemini-300, Inova 400, or Inova 500 instrument with residual solvent signal as the internal standard:  $\text{CDCl}_3$  ( $\delta$  7.26 for proton,  $\delta$  77.16 for carbon). Infrared spectra were recorded on a Perkin-Elmer FT-IR instrument. UV–vis–near-IR spectra were recorded on a Perkin-Elmer Lambda 12 spectrophotometer. Elemental analyses were conducted by Atlantic Microlabs, Inc. Q-TOF 2 electrospray and ESI mass spectrometry were conducted at the Campus Chemical Instrumentation Center of The Ohio State University (Columbus, OH) and the Instrument Center of the Department of Chemistry at the University at Buffalo. Compounds **1**, **12–15**, **21–23**, **45**, and **46** were prepared, as previously described<sup>12–14</sup> and compound **2**, prepared in our earlier works,<sup>12</sup> was used as the disodium salt. In biological studies, core-modified porphyrins **1–11** were dissolved in DMSO to make a stock solution at  $2 \times 10^{-3}$  M. The stock solutions were then used after appropriate dilutions with sterilized doubly distilled water.

### 4.2. Synthesis

**4.2.1. General method for the preparation of 2,5-bis(aryl-1-hydroxymethyl)thiophenes (16–18).** Compounds **17** and **18** were prepared as described for the preparation of **16**.

**4.2.2. 2,5-Bis[1-(4-isopropyl)phenyl-1-hydroxymethyl]thiophene (16).** Thiophene (4.2 g, 50 mmol) was added to a solution of *n*-butyllithium (69 mL of a 1.6 M solution in hexanes, 110 mmol) and TMEDA (17 mL, 115 mmol) in 200 mL hexanes under an Ar atmosphere. The reaction mixture was heated at reflux for 1 h, cooled to ambient temperature, and transferred via a cannula to a pres-

sure-equalizing addition funnel. This dilithiothiophene suspension was then added dropwise to a solution of 4-isopropylbenzaldehyde (14 g, 95 mmol) in 200 mL of anhydrous THF cooled to 0 °C, which had been degassed with Ar for 15 min. After the addition was complete, the mixture was warmed to ambient temperature, 300 mL of NH<sub>4</sub>Cl (aqueous 1 M solution) was added, and the organic phase was separated. The aqueous phase was extracted with ether (3× 300 mL). The combined organic extracts were washed with water (3× 300 mL) and brine (300 mL), dried over MgSO<sub>4</sub>, and concentrated. The crude product was purified by a silica column with the mixture of hexanes and ethyl acetate to give 14 g (73%) of **16** as a light yellow oil. IR (film): 3415, 3036, 2940, 2870, 1699 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 1.17 (12H, d, *J* = 7.0 Hz), 3.16–3.20 (2H, m), 5.85 (2H, s), 6.62 (2H, s), 7.14 (4H, d, *J* = 8.0 Hz), 7.27 (4H, d, *J* = 7.5 Hz); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>): δ 24.10, 33.96, 72.62, 124.42, 124.45, 126.42, 126.47, 126.73, 140.47, 148.26, 148.85; High Resolution Q-TOF MS: *m/z* 403.1703 (calcd for C<sub>24</sub>H<sub>28</sub>O<sub>2</sub>S+H, 403.1708).

**4.2.3. 2,5-Bis(1-biphenyl-1-hydroxymethyl)thiophene (17).** Yield: 70%; IR (film): 3392, 3030, 1732, 1698 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): δ 5.44 (2H, s), 6.70 (2H, s), 7.28–7.35 (2H, m), 7.38–7.52 (8H, m), 7.54–7.62 (8H, m); <sup>13</sup>C NMR (126 MHz, 1:1 CDCl<sub>3</sub>/CD<sub>3</sub>OD): δ 81.41, 81.45, 125.14, 127.09, 127.11, 127.15, 127.33, 127.39, 127.44, 127.47, 127.51, 128.88, 140.02, 140.06, 140.84, 141.08, 146.29, 146.33; High Resolution ESI MS: *m/z* 448.1481 (calcd for C<sub>30</sub>H<sub>24</sub>O<sub>2</sub>S, 448.1492).

**4.2.4. 2,5-Bis[1-(4-triisobutylsilyloxy)phenyl-1-hydroxymethyl]thiophene (18).** Yield: 58%; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 1.31 (18H, d, *J* = 6.9 Hz), 1.42–1.50 (6H, br s), 6.13 (2H, s), 6.88 (2H, s), 7.07 (4H, d, *J* = 8.4 Hz), 7.48 (4H, d, *J* = 7.5 Hz); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ .83, 18.06, 72.46, 120.03, 124.44, 127.73, 135.60, 148.50, 156.10. High Resolution ESI MS: *m/z* 663.3344 (calcd for C<sub>36</sub>H<sub>56</sub>O<sub>4</sub>SSi<sub>2</sub>+Na, 663.3330).

**4.2.5. 2-(1-Hydroxy-1-phenylmethyl)-5-[1-(4-fluorophenyl)-1-hydroxymethyl]thiophene (44).** 2-[1-(*tert*-Butyldimethylsilyloxy)-1-phenylmethyl]thiophene<sup>14</sup> (**43**, 6.0 g, 20 mmol) was added to a solution of *n*-butyllithium (14 mL of 1.6 M in hexanes, 22 mmol) and TMEDA (3.6 mL, 24 mmol) in 150 mL of hexanes under an Ar atmosphere. The reaction mixture was stirred at ambient temperature for 30 min and transferred via cannula to a pressure-equalizing addition funnel. The suspension of 2-lithio **43** was then added dropwise to a solution of 4-fluorobenzaldehyde (2.1 mL, 20 mmol) in 150 mL of anhydrous THF at 0 °C, which was degassed with Ar for 15 min. After the addition was complete, the mixture was warmed to ambient temperature, 300 mL of a 1 M solution of NH<sub>4</sub>Cl was added, and the organic phase was separated. The aqueous phase was extracted with ether (3× 200 mL). The combined organic extracts were washed with water (3× 200 mL) and brine (200 mL), dried over MgSO<sub>4</sub>, and concentrated to give a yellow oil. The oil was dissolved in a 1 M solution of Bu<sub>4</sub>NF in THF (95 mL, 95 mmol) and stirred at ambient temperature for 1 h at

which point 100 mL of saturated aqueous NH<sub>4</sub>Cl was added. The resulting mixture was extracted with ether (4× 100 mL). The combined organic extracts were washed with water (3× 200 mL) and brine (200 mL), dried over MgSO<sub>4</sub>, and concentrated to give the crude diol. The crude diol was purified by column chromatography on SiO<sub>2</sub> eluted with a mixture of hexanes and ethyl acetate to give 3.5 g (56%) of **44** as a light yellow oil. IR (film): 3406, 3063, 2874, 1700, 1604 cm<sup>-1</sup>; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 6.22 (2H, s), 6.97 (2H, s), 6.78 (1H, d, *J* = 3.0 Hz), 6.85 (1H, t, *J* = 4.8 Hz), 6.98 (2H, t, *J* = 8.4 Hz), 7.22 (1H, d, *J* = 4.8 Hz), 7.44 (2H, dd, *J*<sub>1</sub> = 8.3, *J*<sub>2</sub> = 5.7); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 71.92, 72.62, 115.47 (d, *J* = 22), 124.60, 126.41, 128.16 (d, *J* = 8), 128.19, 128.69, 138.80, 142.94, 148.02, 148.38, 162.51 (d, *J* = 244), 164.14; High Resolution Q-TOF MS: *m/z* 337.0693 (calcd for C<sub>18</sub>H<sub>15</sub>FN<sub>2</sub>O<sub>2</sub>S+Na, 337.0674).

**4.2.6. 2-(1-Hydroxy-1-phenylmethyl)-5-[1-(4-trifluoromethylphenyl)-1-hydroxymethyl]thiophene (45).** Compound **45** was prepared with the same method as that used for the preparation of **44**. Yield: 57%; IR (film): 3428, 3063, 2874, 1700, 1620 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ 5.82 (1H, s), 5.89 (1H, s), 6.21 (1H, s), 6.42 (1H, s), 6.68 (1H, d, *J* = 10.4 Hz), 6.71 (1H, d, *J* = 11.6 Hz), 7.18–7.25 (1H, m), 7.30 (2H, br s), 7.35 (2H, br s), 7.60 (2H, br s), 7.66 (2H, br s); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ 70.50, 71.13, 123.54 (123.90), 125.48 (125.52), 126.31 (126.39), 127.02 (127.08), 127.59 (127.64), 128.56, 145.02 (145.08), 148.35 (148.52), 149.67, 149.91 (150.12); High Resolution Q-TOF MS: *m/z* 387.0635 (calcd for C<sub>19</sub>H<sub>15</sub>F<sub>3</sub>N<sub>2</sub>O<sub>2</sub>S+Na, 387.0643).

**4.2.7. Diethyl 2,5-bis[1-(4-carboxylatmethoxyphenyl)-1-pyrrolomethyl]thiophene (23).** Diol **20** (3.2 g, 5.8 mmol) was dissolved in excess pyrrole (16 mL) and the resulting solution was degassed with Ar. Boron trifluoride etherate was added (0.10 mL, 1.1 mmol) and the resulting mixture was stirred for 1 h at ambient temperature. The reaction was stopped by the addition of CH<sub>2</sub>Cl<sub>2</sub> (100 mL), followed by 40% NaOH (50 mL). The organic layer was separated, washed with water (3× 150 mL) and brine (150 mL), dried over MgSO<sub>4</sub>, and concentrated. The excess pyrrole was removed at reduced pressure at ambient temperature. The residual oil was purified via chromatography on SiO<sub>2</sub> eluted with the mixture of hexanes and ethyl acetate to give 2.7 g (63%) of **23** as a yellow oil. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 1.10 (18H, d, *J* = 7.5 Hz), 1.40–1.48 (6H, s), 5.51 (2H, s), 5.90 (2H, s), 6.14 (2H, br s), 6.58 (2H, s), 6.68 (2H, s), 6.82 (4H, d, *J* = 8.5 Hz), 7.07 (4H, d, *J* = 8.5 Hz), 7.89 (2H, br s); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>): δ 12.76, 18.05, 45.33, 107.45, 108.35, 117.20, 120.01, 125.17, 129.44, 133.62, 135.26, 146.46, 155.14; High Resolution ESI MS: *m/z* 761.3958 (calcd for C<sub>44</sub>H<sub>62</sub>N<sub>2</sub>O<sub>2</sub>SSi<sub>2</sub>+Na, 761.3963).

**4.2.8. General method for the preparation of 5,20-diaryl-10,15-bis(4-methoxyphenyl)-21,23-dithiaporphyrins (24–30, 46, 47).** Compounds, **24–30**, **46**, and **47**, were prepared with similar methods described for the preparation of **24**.

**4.2.9. 5,20-Bis(4-chlorophenyl)-10,15-bis(4-methoxyphenyl)-21,23-dithiaporphyrin (24).** Diol **12** (3.0 g, 0.8 mmol), 2,5-bis[1-(4-chlorophenyl)-1-pyrrolomethyl]thiophene (**22**, 3.8 g, 0.8 mmol), and 2,3,5,6-tetrachloro-1,4-benzoquinone (TCBQ, 6.1 g, 25 mmol) were dissolved in 600 mL CH<sub>2</sub>Cl<sub>2</sub>. Boron trifluoride etherate (0.57 mL, 0.45 mmol) was added and the reaction mixture was stirred for 0.5 h in the dark. The reaction mixture was concentrated and the residue was redissolved in minimal CH<sub>2</sub>Cl<sub>2</sub>. The crude product was purified via chromatography on basic alumina eluted with CH<sub>2</sub>Cl<sub>2</sub>. Dithiaporphyrin **24** was isolated as the first red band. The crude product was washed with acetone to give 0.32 g (5%) of **24** as a purple solid. Mp: >300 °C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 4.11 (6H, s), 7.36 (4H, d, *J* = 8.4 Hz), 7.80 (4H, d, *J* = 8.0 Hz), 8.168 (4H, d, *J* = 8.4 Hz), 8.169 (4H, d, *J* = 8.0 Hz), 8.64 (2H, d, *J* = 4.4 Hz), 8.72 (2H, d, *J* = 4.4 Hz), 9.63 (2H, s), 9.73 (2H, s); <sup>13</sup>C NMR (75 MHz, 1:1 CDCl<sub>3</sub>/CD<sub>3</sub>OD): δ 55.79, 113.29, 127.93, 132.36, 133.70, 134.22, 134.63, 134.80, 135.13, 135.21, 135.32, 135.63, 135.86, 139.86, 147.59, 148.60, 156.38, 156.96, 160.04; High Resolution Q-TOF MS: *m/z* 777.1204 (calcd for C<sub>46</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>+H, 777.1204).

**4.2.10. 5,20-Bis(4-trifluoromethylphenyl)-10,15-bis(4-methoxyphenyl)-21,23-dithiaporphyrin (25).** Yield: 3%; mp: >300 °C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 4.10 (6H, s), 7.37 (4H, d, *J* = 7.6 Hz), 8.09 (4H, d, *J* = 7.2 Hz), 8.18 (4H, d, *J* = 8.0 Hz), 8.36 (4H, d, *J* = 7.2 Hz), 8.60 (2H, d, *J* = 4.4 Hz), 8.74 (2H, d, *J* = 4.0 Hz), 9.59 (2H, s), 9.75 (2H, s); High Resolution Q-TOF MS: *m/z* 845.1735 (calcd for C<sub>48</sub>H<sub>30</sub>F<sub>6</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>+H, 845.1731).

**4.2.11. 5,20-Bis(4-dimethylaminophenyl)-10,15-bis(4-methoxyphenyl)-21,23-dithiaporphyrin (26).** Yield: 6.4%; mp: >300 °C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 3.25 (12H, s), 4.10 (6H, s), 7.17 (4H, d, *J* = 8.8 Hz), 7.35 (4H, d, *J* = 8.8 Hz), 8.15 (4H, d, *J* = 5.2 Hz), 8.18 (2H, d, *J* = 5.2 Hz), 8.66 (2H, d, *J* = 4.4 Hz), 8.75 (2H, d, *J* = 4.8 Hz), 9.64 (2H, s), 9.76 (2H, s); High Resolution Q-TOF MS: *m/z* 795.2834 (calcd for C<sub>40</sub>H<sub>42</sub>N<sub>4</sub>O<sub>2</sub>S<sub>2</sub>+H, 795.2827).

**4.2.12. 5,20-Bis(4-isobutylphenyl)-10,15-bis(4-methoxyphenyl)-21,23-dithiaporphyrin (27).** Yield: 30%; mp: >300 °C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 1.56 (12H, d, *J* = 6.8 Hz), 3.26–3.30 (2H, m), 4.10 (6H, s), 7.35 (4H, d, *J* = 8.4 Hz), 7.67 (4H, d, *J* = 7.6 Hz), 8.18 (8H, d, *J* = 8.0 Hz), 8.45 (2H, d, *J* = 4.4 Hz), 8.73 (2H, d, *J* = 4.4 Hz), 9.71 (2H, s), 9.72 (2H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 24.40, 34.28, 55.72, 113.15, 125.67, 133.19, 133.95, 134.26, 134.53, 134.67, 135.55, 138.86, 148.02, 148.73, 156.62, 156.71, 159.84. High Resolution Q-TOF MS: *m/z* 793.2938 (calcd for C<sub>52</sub>H<sub>44</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>+H, 793.2922).

**4.2.13. 5,20-Di(biphenyl)-10,15-bis(4-methoxyphenyl)-21,23-dithiaporphyrin (28).** Yield: 18%; mp: 175–177 °C; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 4.12 (6H, s), 7.37 (4H, d, *J* = 7.5 Hz), 7.48–7.52 (2H, m), 7.61 (4H, t, *J* = 7.5 Hz), 7.93 (4H, d, *J* = 7.5 Hz), 8.06 (4H, d, *J* = 7.5 Hz), 8.20 (4H, d, *J* = 7.5 Hz), 8.34 (4H, d, *J* = 7.5 Hz), 8.73 (2H, d, *J* = 4.5 Hz), 8.76 (2H, d,

*J* = 4.5 Hz), 9.73 (2H, s), 9.77 (2H, s); High Resolution ESI MS: *m/z* 861.2592 (calcd for C<sub>58</sub>H<sub>40</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>+H, 861.2604).

**4.2.14. Diethyl 5,20-bis(4-methoxyphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (29).** Yield: 18%; mp: 117–119 °C; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 1.42 (6H, t, *J* = 7.0 Hz), 4.10 (6H, s), 4.42 (4H, q, *J* = 7.0 Hz), 4.92 (4H, s), 7.36 (8H, d, *J* = 7.0 Hz), 8.17 (8H, d, *J* = 7.5 Hz), 8.67 (2H, d, *J* = 5.0 Hz), 8.72 (2H, d, *J* = 4.5 Hz), 9.68 (2H, s), 9.70 (2H, s); High Resolution ESI MS: *m/z* 913.2645 (calcd for C<sub>54</sub>H<sub>44</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub>+H, 913.2612).

**4.2.15. Diethyl 5,20-bis(4-triisobutylsilyloxyphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (30).** Yield: 32%; mp: 195–198 °C; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ 1.29 (36H, d, *J* = 7.5 Hz), 1.42 (6H, t, *J* = 7.0 Hz), 1.45–1.51 (6H, s), 4.42 (4H, q, *J* = 7.0 Hz), 4.93 (4H, s), 7.32–7.39 (8H, s), 8.09 (4H, d, *J* = 6.5 Hz), 8.18 (4H, d, *J* = 7.0 Hz), 8.66–8.70 (4H, s), 9.68 (2H, s), 9.71 (2H, s); High Resolution ESI MS: *m/z* 1197.4987 (calcd for C<sub>70</sub>H<sub>80</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub>Si<sub>2</sub>+H, 1197.4967).

**4.2.16. 5-Phenyl-20-(4-fluorophenyl)-10,15-bis(4-methoxyphenyl)-21,23-dithiaporphyrin (46).** Yield: 5%; mp: >300 °C; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ 4.10 (6H, s), 7.36 (4H, d, *J* = 8.1 Hz), 7.45 (2H, t, *J* = 8.1 Hz), 7.81 (3H, br s), 8.18 (4H, d, *J* = 8.1 Hz), 8.16–8.30 (4H, m), 8.60–8.76 (4H, m), 9.63 (1H, d, *J* = 4.8 Hz), 9.69 (1H, d, *J* = 5.1 Hz), 9.73 (2H, br s); High Resolution Q-TOF MS: *m/z* 727.1874 (calcd for C<sub>46</sub>H<sub>31</sub>FN<sub>2</sub>O<sub>2</sub>S<sub>2</sub>+H, 727.1889).

**4.2.17. 5-Phenyl-20-(4-trifluoromethylphenyl)-10,15-bis(4-methoxyphenyl)-21,23-dithiaporphyrin (47).** Yield: 7%; mp: 125–127 °C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 11 (6H, s), 7.37 (4H, d, *J* = 8.4 Hz), 7.79–7.85 (3H, m), 8.08 (2H, d, *J* = 8.0 Hz), 8.18 (4H, d, *J* = 8.0 Hz), 8.22–8.27 (2H, m), 8.37 (2H, d, *J* = 8.0 Hz), 8.60 (1H, d, *J* = 4.4 Hz), 8.68 (1H, d, *J* = 4.4 Hz), 8.72 (1H, d, *J* = 4.4 Hz), 8.74 (1H, d, *J* = 4.4 Hz), 9.58 (1H, d, *J* = 5.2 Hz), 9.68 (1H, d, *J* = 5.2 Hz), 9.74 (2H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ 55.78, 111.07, 113.27, 124.57, 127.63, 128.25, 130.82, 131.53, 133.71, 133.99, 134.33, 134.62, 134.79, 134.95, 135.17, 135.61, 135.75, 135.85, 141.32, 145.20, 147.47, 147.70, 148.48, 148.67, 150.65, 155.96, 156.80, 160.01, 160.92; High Resolution Q-TOF MS: *m/z* 777.1868 (calcd for C<sub>47</sub>H<sub>31</sub>F<sub>3</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub>+H, 777.1857).

**4.2.18. General method for the preparation of 5,20-diaryl-10,15-bis(4-hydroxyphenyl)-21,23-dithiaporphyrins (31–35, 48, 49).** Compounds, **31–35**, **48**, and **49**, were prepared, as described for the preparation of **31**.

**4.2.19. 5,20-Bis(4-chlorophenyl)-10,15-bis(4-hydroxyphenyl)-21,23-dithiaporphyrin (31).** Dithiaporphyrin **24** (0.27 g, 0.35 mmol) was dissolved in 50 mL CH<sub>2</sub>Cl<sub>2</sub> and BBr<sub>3</sub> (0.32 mL, 3.5 mmol) was added at 0 °C. The resulting solution was stirred for 5 h at ambient temperature. The reaction mixture was added to 150 mL EtOAc and 150 mL of saturated NaHCO<sub>3</sub>. The organic



layer was separated and washed three times with 150 mL of brine, dried over  $\text{MgSO}_4$ , and concentrated. The crude solid was washed with 25% EtOAc/hexanes several times to give 0.23 g (88%) of **31** as a dark blue solid. Mp:  $>300^\circ\text{C}$ ;  $^1\text{H}$  NMR (400 MHz, 1:1  $\text{CDCl}_3/\text{CD}_3\text{OD}$ ):  $\delta$  7.18 (4H, d,  $J = 8.4$  Hz), 7.69 (4H, d,  $J = 8.0$  Hz), 7.98 (4H, d,  $J = 8.4$  Hz), 8.05 (4H, d,  $J = 8.0$  Hz), 8.52 (2H, d,  $J = 4.4$  Hz), 8.64 (2H, d,  $J = 4.4$  Hz), 9.53 (2H, s), 9.67 (2H, s);  $^{13}\text{C}$  NMR (75 MHz, 1:1  $\text{CDCl}_3/\text{CD}_3\text{OD}$ ):  $\delta$  114.53, 127.67, 132.00, 132.34, 133.79, 134.55, 134.98, 135.08, 135.60, 135.79, 139.55, 147.16, 148.40, 156.13, 156.81, 157.32; High Resolution Q-TOF MS:  $m/z$  749.0891 (calcd for  $\text{C}_{44}\text{H}_{26}\text{Cl}_2\text{N}_2\text{O}_2\text{S}_2 + \text{H}$ , 749.0888).

**4.2.20. 5,20-Bis(4-trifluoromethylphenyl)-10,15-bis(4-hydroxyphenyl)-21,23-dithiaporphyrin (32).** Yield: 88%; mp:  $>300^\circ\text{C}$ ;  $^1\text{H}$  NMR (400 MHz, 1:1  $\text{CDCl}_3/\text{CD}_3\text{OD}$ ):  $\delta$  7.23–7.33 (4H, m), 8.00–8.13 (8H, m), 8.31 (4H, d,  $J = 7.6$  Hz), 8.54 (2H, d,  $J = 4.4$  Hz), 8.72 (2H, d,  $J = 4.4$  Hz), 9.56 (2H, d,  $J = 3.2$  Hz), 9.75 (2H, d,  $J = 2.4$  Hz); High Resolution Q-TOF MS:  $m/z$  817.1425 (calcd for  $\text{C}_{46}\text{H}_{26}\text{F}_6\text{N}_2\text{O}_6\text{S}_2 + \text{H}$ , 817.1418).

**4.2.21. 5,20-Bis(4-dimethylaminophenyl)-10,15-bis(4-hydroxyphenyl)-21,23-dithiaporphyrin (33).** Yield: 83%; mp:  $>300^\circ\text{C}$ ;  $^1\text{H}$  NMR (400 MHz, 1:1  $\text{CDCl}_3/\text{CD}_3\text{OD}$ ):  $\delta$  3.21 (12H, s), 7.16 (4H, d,  $J = 7.6$  Hz), 7.25 (4H, d,  $J = 8.0$  Hz), 8.02 (4H, d,  $J = 5.2$  Hz), 8.06 (4H, d,  $J = 5.2$  Hz), 8.59 (2H, s), 8.62 (2H, s), 9.65 (2H, s), 9.69 (2H, s); HI ESI MS: 767.2521 (calcd for  $\text{C}_{48}\text{H}_{38}\text{N}_4\text{O}_2\text{S}_2 + \text{H}$ , 767.2514).

**4.2.22. 5,20-Bis(4-isobutylphenyl)-10,15-bis(4-hydroxyphenyl)-21,23-dithiaporphyrin (34).** Yield: 95%; mp: 234–236  $^\circ\text{C}$ ;  $^1\text{H}$  NMR (500 MHz, acetone- $d_6$ ):  $\delta$  1.54 (12H, d,  $J = 7.0$  Hz), 3.21–3.34 (2H, m), 7.40 (4H, d,  $J = 8.0$  Hz), 7.75 (4H, d,  $J = 7.5$  Hz), 8.15 (4H, d,  $J = 8.5$  Hz), 8.17 (4H, d,  $J = 8.0$  Hz), 8.66 (2H, d,  $J = 4.0$  Hz), 8.74 (2H, d,  $J = 4.5$  Hz), 9.73 (2H, s), 9.83 (2H, s);  $^{13}\text{C}$  NMR (75 MHz, 1:1:1  $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{DMSO}-d_6$ ):  $\delta$  24.33, 34.00, 115.00, 125.79, 131.70, 133.87, 134.34, 134.46, 134.61, 135.67, 138.31, 147.36, 147.86, 148.69, 156.10, 156.32, 158.10; High Resolution Q-TOF MS:  $m/z$  765.2614 (calcd for  $\text{C}_{50}\text{H}_{40}\text{N}_2\text{O}_2\text{S}_2 + \text{H}$ , 765.2609).

**4.2.23. 5,20-Di(biphenyl)-10,15-bis(4-hydroxyphenyl)-21,23-dithiaporphyrin (35).** Yield: 86%; mp:  $>300^\circ\text{C}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.28 (4H, br s), 7.45 (2H, br s), 7.55 (4H, br s), 7.86 (4H, br s), 7.93–8.08 (8H, m), 8.18 (4H, br s), 8.62 (4H, br s), 9.68 (4H, br s); High Resolution ESI MS:  $m/z$  833.2274 (calcd for  $\text{C}_{56}\text{H}_{36}\text{N}_2\text{O}_2\text{S}_2 + \text{H}$ , 833.2291).

**4.2.24. 5-Phenyl-20-(4-fluorophenyl)-10,15-bis(4-hydroxyphenyl)-21,23-dithiaporphyrin (48).** Yield: 93%; mp:  $>300^\circ\text{C}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.29 (4H, d,  $J = 8.0$  Hz), 7.49–7.57 (3H, m), 7.81 (2H, br s), 8.08 (4H, d,  $J = 8.5$  Hz), 8.18–8.24 (4H, m), 8.63 (1H, d,  $J = 5.0$  Hz), 8.65 (1H, d,  $J = 4.5$  Hz), 7.59 (2H, t,  $J = 10.5$  Hz), 9.65 (1H, d,  $J = 5.0$  Hz), 9.69 (1H, d,  $J = 5.0$  Hz), 9.78 (2H, s); High Resolution Q-TOF MS:  $m/z$  699.1519 (calcd for  $\text{C}_{44}\text{H}_{27}\text{FN}_2\text{O}_2\text{S}_2 + \text{H}$ , 699.1498).

**4.2.25. 5-Phenyl-20-(4-trifluoromethylphenyl)-10,15-bis(4-hydroxyphenyl)-21,23-dithiaporphyrin (49).** Yield: 93%; mp:  $>300^\circ\text{C}$ ;  $^1\text{H}$  NMR (400 MHz, 1:1:1  $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{DMSO}-d_6$ ):  $\delta$  7.03 (4H, d,  $J = 8.4$  Hz), 7.51–7.58 (3H, m), 7.78 (2H, d,  $J = 8.4$  Hz), 7.81 (4H, d,  $J = 8.0$  Hz), 8.16–8.22 (2H, m), 8.08 (2H, d,  $J = 8.0$  Hz), 8.30 (1H, d,  $J = 4.8$  Hz), 8.36 (1H, d,  $J = 4.4$  Hz), 8.44 (1H, d,  $J = 4.4$  Hz), 8.46 (1H, d,  $J = 4.8$  Hz), 9.30 (1H, d,  $J = 5.2$  Hz), 9.39 (1H, d,  $J = 5.2$  Hz), 9.50 (2H, s); High Resolution Q-TOF MS:  $m/z$  749.1551 (calcd for  $\text{C}_{45}\text{H}_{27}\text{F}_3\text{N}_2\text{O}_2\text{S}_2 + \text{H}$ , 749.1544).

**4.2.26. General method for the preparation of diethyl 5,20-diaryl-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrins (36–40, 50, 51).** Compounds, **36–40**, **50**, and **51**, were prepared as described for the preparation of **36**.

**4.2.27. Diethyl 5,20-bis(4-chlorophenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (36).** Dithiaporphyrin **31** (0.20 g, 0.27 mmol),  $\text{K}_2\text{CO}_3$  (1.8 g, 13 mmol), and ethyl bromoacetate (2.96 mL, 27 mmol) in 50 mL acetone were heated at reflux for 10 h. The reaction mixture was cooled to ambient temperature and the  $\text{K}_2\text{CO}_3$  was removed by filtration. The filter cake was washed with acetone until the filtrate became colorless. The combined filtrates were concentrated. The crude product was washed with MeOH to give 0.20 g (81%) of **36** as a purple solid. Mp: 212–214  $^\circ\text{C}$ ;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.45 (6H, t,  $J = 7.6$  Hz), 4.44 (4H, q,  $J = 6.8$  Hz), 4.92 (4H, s), 7.36 (4H, d,  $J = 8.4$  Hz), 7.80 (4H, d,  $J = 8.0$  Hz), 8.17 (4H, d,  $J = 8.0$  Hz), 8.17 (4H, d,  $J = 8.0$  Hz), 8.66 (2H, d,  $J = 4.4$  Hz), 8.72 (2H, d,  $J = 4.8$  Hz), 9.65 (2H, s), 9.73 (2H, s);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  14.43, 61.73, 65.86, 113.97, 127.88, 132.45, 134.17, 134.29, 134.66, 134.78, 134.88, 135.02, 134.27, 135.54, 135.79, 139.73, 147.62, 148.44, 156.37, 156.84, 158.28; High Resolution Q-TOF MS:  $m/z$  921.1629 (calcd for  $\text{C}_{52}\text{H}_{38}\text{Cl}_2\text{N}_2\text{O}_6\text{S}_2 + \text{H}$ , 921.1626).

**4.2.28. Diethyl 5,20-bis(4-trifluoromethylphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (37).** Yield: 86%; mp: 133–135  $^\circ\text{C}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.38 (6H, t,  $J = 7.0$  Hz), 4.38 (4H, q,  $J = 7.0$  Hz), 4.88 (4H, s), 7.40 (4H, d,  $J = 9.0$  Hz), 8.12 (4H, d,  $J = 7.5$  Hz), 8.20 (4H, d,  $J = 8.0$  Hz), 8.38 (4H, d,  $J = 8.0$  Hz), 8.63 (2H, d,  $J = 4.5$  Hz), 8.75 (2H, d,  $J = 4.0$  Hz), 9.62 (2H, s), 9.76 (2H, s); High Resolution Q-TOF MS:  $m/z$  989.2190 (calcd for  $\text{C}_{54}\text{H}_{38}\text{F}_6\text{N}_2\text{O}_6\text{S}_2 + \text{H}$ , 989.2922).

**4.2.29. Diethyl 5,20-bis(4-dimethylaminophenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (38).** Yield: 68%; mp:  $>300^\circ\text{C}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.42 (6H, t,  $J = 7.0$  Hz), 3.25 (12H, s), 4.42 (4H, q,  $J = 7.5$  Hz), 4.92 (4H, s), 7.18 (4H, d,  $J = 7.0$  Hz), 7.35 (4H, d,  $J = 8.5$  Hz), 8.16 (8H, t,  $J = 8.5$  Hz), 8.63 (2H, d,  $J = 4.5$  Hz), 8.74 (2H, d,  $J = 4.5$  Hz), 9.62 (2H, s), 9.76 (2H, s); High Resolution ESI MS:  $m/z$  939.3260 (calcd for  $\text{C}_{56}\text{H}_{50}\text{N}_4\text{O}_6\text{S}_2 + \text{H}$ , 939.3250).

**4.2.30. Diethyl 5,20-bis(4-isopropylphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (39).** Yield: 82%; mp: 180–182  $^\circ\text{C}$ ;  $^1\text{H}$  NMR (400 MHz,

CDCl<sub>3</sub>):  $\delta$  1.43 (6H, t,  $J$  = 7.2 Hz), 1.56 (12H, d,  $J$  = 6.8 Hz), 3.23–3.34 (2H, m), 4.43 (4H, q,  $J$  = 6.8 Hz), 4.93 (4H, s), 7.36 (4H, d,  $J$  = 8.4 Hz), 7.67 (4H, d,  $J$  = 8.0 Hz), 8.17 (4H, d,  $J$  = 8.0 Hz), 8.19 (4H, d,  $J$  = 8.4 Hz), 8.68 (2H, d,  $J$  = 4.4 Hz), 8.72 (2H, d,  $J$  = 4.4 Hz), 9.68 (2H, s), 9.72 (2H, s); <sup>13</sup>C NMR (75 MHz, 1:1 CDCl<sub>3</sub>/DMSO-*d*<sub>6</sub>):  $\delta$  14.44, 24.40, 34.29, 61.73, 65.93, 111.04, 113.91, 125.68, 133.44, 134.42, 134.54, 134.78, 135.00, 135.34, 135.52, 135.65, 138.82, 148.03, 148.12, 148.78, 156.67, 158.19, 169.16; High Resolution Q-TOF MS:  $m/z$  937.3388 (calcd for C<sub>58</sub>H<sub>52</sub>N<sub>2</sub>O<sub>6</sub>S<sub>2</sub>+H, 937.3345).

**4.2.31. Diethyl 5,20-di(biphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (40).** Yield: 87%; mp: 122–124 °C; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  1.42 (6H, t,  $J$  = 7.0 Hz), 4.42 (4H, q,  $J$  = 7.0 Hz), 4.93 (4H, s), 7.38 (4H, d,  $J$  = 7.5 Hz), 7.49 (2H, t,  $J$  = 7.5 Hz), 7.61 (4H, t,  $J$  = 7.5 Hz), 7.93 (4H, d,  $J$  = 7.0 Hz), 8.06 (4H, d,  $J$  = 7.5 Hz), 8.20 (4H, d,  $J$  = 8.0 Hz), 8.33 (4H, d,  $J$  = 7.5 Hz), 8.71 (2H, d,  $J$  = 4.5 Hz), 8.76 (2H, d,  $J$  = 4.5 Hz), 9.71 (2H, s), 9.78 (2H, s); High Resolution ESI MS:  $m/z$  1005.3010 (calcd for C<sub>64</sub>H<sub>48</sub>N<sub>2</sub>O<sub>6</sub>S<sub>2</sub>+H, 1005.3027).

**4.2.32. Diethyl 5-phenyl-20-(4-fluorophenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (50).** Yield: 81%; mp: 145–147 °C; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  1.43 (6H, t,  $J$  = 7.2 Hz), 4.43 (4H, q,  $J$  = 6.8 Hz), 4.93 (4H, s), 7.37 (4H, d,  $J$  = 8.4 Hz), 7.52 (2H, t,  $J$  = 8.4 Hz), 7.79–7.86 (3H, m), 8.18 (4H, d,  $J$  = 8.4 Hz), 8.18–8.28 (4H, m), 8.65 (1H, d,  $J$  = 4.4 Hz), 8.67–8.72 (3H, m), 9.63 (1H, d,  $J$  = 5.2 Hz), 9.68 (1H, d,  $J$  = 5.2 Hz), 9.70 (2H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$  14.37, 61.68, 64.46, 65.82, 110.97, 113.87, 114.59 (d,  $J$  = 22), 127.52, 128.15, 133.83, 134.24, 134.73, 135.11, 135.47, 141.25, 147.81, 148.18, 156.70, 158.17, 162.97 (d,  $J$  = 320), 169.06; High Resolution Q-TOF MS:  $m/z$  871.2320 (calcd for C<sub>52</sub>H<sub>39</sub>FN<sub>2</sub>O<sub>6</sub>S<sub>2</sub>+H, 871.2312).

**4.2.33. Diethyl 5-phenyl-20-(4-trifluoromethylphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (51).** Yield: 87%; mp: 125–127 °C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  1.44 (6H, t,  $J$  = 6.8 Hz), 4.44 (4H, q,  $J$  = 6.8 Hz), 4.93 (4H, s), 7.38 (4H, d,  $J$  = 4.0 Hz), 7.79–7.89 (3H, m), 8.09 (2H, d,  $J$  = 8.0 Hz), 8.18 (4H, d,  $J$  = 8.0 Hz), 8.23–8.29 (2H, m), 8.37 (2H, d,  $J$  = 8.4 Hz), 8.62 (1H, d,  $J$  = 4.4 Hz), 8.68–8.76 (3H, m), 9.59 (1H, d,  $J$  = 5.2 Hz), 9.70 (1H, d,  $J$  = 4.8 Hz), 9.73 (2H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$  14.43, 61.74, 65.88, 113.96, 124.55, 127.62, 128.27, 131.65, 134.02, 134.09, 134.31, 134.44, 134.73, 134.86, 135.08, 135.55, 135.71, 135.80, 135.91, 141.23, 145.10, 147.51, 147.76, 148.33, 148.52, 155.97, 156.82, 158.29, 169.10; High Resolution Q-TOF MS:  $m/z$  921.2308 (calcd for C<sub>53</sub>H<sub>39</sub>F<sub>3</sub>N<sub>2</sub>O<sub>6</sub>S<sub>2</sub>+H, 921.2280).

**4.2.34. Diethyl 5,20-bis(4-hydroxyphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (41).** Yield: 84%; mp: 172–174 °C; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  1.31 (6H, t,  $J$  = 6.5 Hz), 4.30 (4H, q,  $J$  = 7.0 Hz), 5.09 (4H, s), 7.28 (4H, d,  $J$  = 7.5 Hz), 7.44 (4H, d,  $J$  = 7.5 Hz), 8.06 (4H, d,  $J$  = 8.0 Hz), 8.17 (4H,

d,  $J$  = 7.5 Hz), 8.60 (2H, s), 8.66 (2H, s), 9.70 (2H, s), 9.76 (2H, s), 10.08 (2H, s); High Resolution ESI MS:  $m/z$  885.2299 (calcd for C<sub>52</sub>H<sub>40</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub>+H, 885.2316).

**4.2.35. General method for the preparation of diethyl 5,20-diaryl-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrins (3–11).** Compounds 3–11 were prepared, as described for the preparation of 4.

**4.2.36. 5,20-Bis(4-chlorophenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (4).** Core-modified porphyrin 36 (0.13 g, 0.14 mmol) was dissolved in 20 mL THF and 10 mL of 1 M aqueous NaOH was added. The resulting solution was stirred at ambient temperature for 15 h. The solution was acidified by the addition of 4.3 mL acetic acid. The reaction mixture was diluted with 100 mL H<sub>2</sub>O and the products were extracted with EtOAc (3 × 100 mL). The combined organic extracts were dried over MgSO<sub>4</sub> and concentrated. The crude product was washed with several portions of hexanes/MeOH to give 0.11 g (90%) of 4 as a purple solid. Mp: 210–212 °C; <sup>1</sup>H NMR (400 MHz, 1:1 CDCl<sub>3</sub>/CD<sub>3</sub>OD):  $\delta$  4.93 (4H, s), 7.40 (4H, d,  $J$  = 8.4 Hz), 7.80 (4H, d,  $J$  = 7.6 Hz), 8.12–8.21 (8H, m), 8.65 (2H, d,  $J$  = 4.0 Hz), 8.71 (2H, d,  $J$  = 4.4 Hz), 9.66 (2H, s), 9.75 (2H, s); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): 65.65, 114.17, 128.09, 132.74, 134.46, 134.54, 134.62, 135.04, 135.20, 135.48, 135.60, 135.78, 136.14, 139.80, 147.75, 148.59, 156.60, 157.10, 158.57, 171.57; High Resolution Q-TOF MS:  $m/z$  865.1021 (calcd for C<sub>48</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>6</sub>S<sub>2</sub>+H, 865.1000); Anal. Calcd for C<sub>48</sub>H<sub>30</sub>Cl<sub>2</sub>N<sub>2</sub>O<sub>6</sub>S<sub>2</sub>: C, 66.59; H, 3.49; N, 3.24. Found: C, 66.99; H, 3.82; N, 2.94.

**4.2.37. 5,20-Bis(4-trifluoromethylphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (5).** Yield: 92%; mp: 220–222 °C; <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  4.90 (4H, s), 7.38 (4H, d,  $J$  = 8.4 Hz), 8.05 (8H, t,  $J$  = 6.3 Hz), 8.36 (4H, d,  $J$  = 7.6 Hz), 8.58 (2H, d,  $J$  = 4.4 Hz), 8.69 (2H, d,  $J$  = 4.4 Hz), 9.62 (2H, s), 9.76 (2H, s); High Resolution Q-TOF MS:  $m/z$  933.1529 (calcd for C<sub>50</sub>H<sub>30</sub>F<sub>6</sub>N<sub>2</sub>O<sub>6</sub>S<sub>2</sub>+H, 933.1528); Anal. Calcd for C<sub>50</sub>H<sub>30</sub>F<sub>6</sub>N<sub>2</sub>O<sub>6</sub>S<sub>2</sub>: C, 64.37; H, 3.24; N, 3.00. Found: C, 64.20; H, 3.02; N, 2.96.

**4.2.38. 5,20-Bis(4-methoxyphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (7).** Yield: 80%; mp: >300 °C; <sup>1</sup>H NMR (400 MHz, 1:1 CDCl<sub>3</sub>/CD<sub>3</sub>OD):  $\delta$  4.06 (6H, s), 4.89 (4H, s), 7.33 (4H, d,  $J$  = 8.4 Hz), 7.37 (4H, d,  $J$  = 8.4 Hz), 8.09 (4H, d,  $J$  = 6.8 Hz), 8.11 (4H, d,  $J$  = 6.4 Hz), 8.62 (4H, s), 9.67 (4H, s); High Resolution ESI MS:  $m/z$  857.1994 (calcd for C<sub>50</sub>H<sub>36</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub>+H, 857.1986); Anal. Calcd for C<sub>50</sub>H<sub>36</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub>: C, 70.08; H, 4.23; N, 3.27. Found: C, 70.23; H, 4.00; N, 3.24.

**4.2.39. 5,20-Bis(4-dimethylaminophenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (8).** Yield: 86%; mp: >300 °C; <sup>1</sup>H NMR (400 MHz, 1:1 CDCl<sub>3</sub>/CD<sub>3</sub>OD):  $\delta$  3.28 (12H, s), 4.91 (4H, s), 7.21 (4H, d,  $J$  = 8.4 Hz), 7.41 (4H, d,  $J$  = 6.8 Hz), 8.10 (8H, t,  $J$  = 7.6 Hz), 8.52 (2H, d,  $J$  = 4.0 Hz), 8.62 (2H, d,  $J$  = 4.0 Hz), 9.57 (2H, s), 9.65 (2H, s); <sup>13</sup>C NMR (75 MHz, 1:1 CDCl<sub>3</sub>/CD<sub>3</sub>OD):  $\delta$  29.02, 64.87, 110.95,

113.31, 128.40, 132.21, 133.33, 133.77, 134.17, 134.78, 135.05, 135.32, 146.75, 147.65, 155.56, 155.86, 170.26; High Resolution ESI MS:  $m/z$  883.2625 (calcd for  $C_{52}H_{42}N_4O_6S_2+H$ , 883.2624); Anal. Calcd for  $C_{52}H_{42}N_4O_6S_2$ : C, 70.73; H, 4.79; N, 6.34. Found: C, 70.62; H, 4.70; N, 6.28.

**4.2.40. 5,20-Bis(4-isopropylphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (10).** Yield: 93%; mp: 220–222 °C;  $^1H$  NMR (500 MHz, 1:1  $CDCl_3$ /DMSO- $d_6$ ):  $\delta$  1.49 (12H, d,  $J = 7.0$  Hz), 2.47–2.53 (2H, m), 4.87 (4H, s), 7.36 (4H, d,  $J = 8.5$  Hz), 7.65 (4H, d,  $J = 7.5$  Hz), 8.10 (4H, d,  $J = 8.0$  Hz), 8.11 (4H, d,  $J = 8.5$  Hz), 8.12–8.21 (8H, m), 8.62 (2H, d,  $J = 4.5$  Hz), 8.64 (2H, d,  $J = 4.5$  Hz), 9.68 (2H, s), 9.68 (2H, s);  $^{13}C$  NMR (75 MHz, 1:1  $CDCl_3$ /DMSO- $d_6$ ):  $\delta$  24.55, 34.20, 65.59, 114.27, 126.05, 133.90, 134.06, 134.44, 134.56, 134.78, 135.00, 135.63, 135.85, 135.98, 138.42, 147.76, 147.84, 148.94, 156.40, 156.46, 158.58, 170.79; High Resolution Q-TOF MS:  $m/z$  881.2663 (calcd for  $C_{54}H_{44}N_2O_6S_2+H$ , 881.2719); Anal. Calcd for  $C_{54}H_{44}N_2O_6S_2$ : C, 73.61; H, 5.03; N, 3.18. Found: C, 73.34; H, 5.15; N, 3.08.

**4.2.41. 5,20-Di(biphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (11).** Yield: 85%; mp: >300 °C;  $^1H$  NMR (500 MHz, DMSO- $d_6$ ):  $\delta$  4.97 (4H, s), 7.42 (4H, d,  $J = 8.0$  Hz), 7.49 (2H, t,  $J = 7.0$  Hz), 7.59 (4H, t,  $J = 7.0$  Hz), 7.94 (4H, d,  $J = 7.0$  Hz), 8.09 (4H, d,  $J = 7.5$  Hz), 8.17 (4H, d,  $J = 8.0$  Hz), 8.26 (4H, d,  $J = 7.5$  Hz), 8.65 (4H, s), 9.76 (4H, d,  $J = 4.5$  Hz), 13.22 (2H, s); High Resolution Q-TOF MS:  $m/z$  949.2401 (calcd for  $C_{60}H_{40}N_2O_6S_2+H$ , 949.2397); Anal. Calcd for  $C_{60}H_{40}N_2O_6S_2$ : C, 75.93; H, 4.25; N, 2.95. Found: C, 76.28; H, 4.24; N, 2.86.

**4.2.42. 5,20-Bis(4-hydroxyphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (9).** Yield: 80%; mp: >300 °C;  $^1H$  NMR (500 MHz, DMSO- $d_6$ ):  $\delta$  4.98 (4H, s), 7.28 (4H, d,  $J = 8.0$  Hz), 7.42 (4H, d,  $J = 8.0$  Hz), 8.06 (4H, d,  $J = 8.0$  Hz), 8.17 (4H, d,  $J = 7.5$  Hz), 8.60 (2H, d,  $J = 4.0$  Hz), 8.66 (2H, d,  $J = 4.5$  Hz), 9.71 (2H, s), 9.75 (2H, s), 10.07 (2H, s), 13.20 (2H, s); High Resolution Q-TOF MS:  $m/z$  829.1673 (calcd for  $C_{48}H_{32}N_2O_8S_2+H$ , 829.1678); Anal. Calcd for  $C_{48}H_{32}N_2O_8S_2$ : C, 69.55; H, 3.89; N, 3.38. Found: C, 69.23; H, 3.83; N, 3.33.

**4.2.43. 5-Phenyl-20-(4-fluorophenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (3).** Yield: 88%; mp: 218–220 °C;  $^1H$  NMR (400 MHz, 1:1  $CDCl_3$ /CD $_3$ OD):  $\delta$  4.85 (4H, s), 7.33 (4H, d,  $J = 6.4$  Hz), 7.43–7.45 (2H, m), 7.73–7.79 (3H, m), 8.11 (4H, d,  $J = 6.0$  Hz), 8.13–8.20 (4H, m), 8.60–8.67 (3H, m), 9.57–9.61 (1H, m), 9.62–9.66 (1H, m), 9.66–9.70 (2H, m);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ ):  $\delta$  65.27, 113.73, 114.42 (d,  $J = 22$ ), 127.38, 128.06, 132.47, 133.78, 133.83, 134.04, 134.35, 134.48, 134.57, 135.33, 135.42, 135.50, 136.99, 140.95, 147.58, 147.99, 156.31, 156.44, 156.57, 158.08, 163.02 (d,  $J = 246$ ), 171.11; High Resolution Q-TOF MS:  $m/z$  815.1666 (calcd for  $C_{48}H_{31}F_1N_2O_6S_2+H$ , 815.1686); Anal. Calcd for  $C_{48}H_{31}F_1N_2O_6S_2$ : C, 70.75; H, 3.83; N, 3.44. Found: C, 71.13; H, 3.92; N, 3.46.

**4.2.44. 5-Phenyl-20-(4-trifluoromethylphenyl)-10,15-bis(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (6).** Yield: 90%; mp: 208–210 °C;  $^1H$  NMR (400 MHz, 1:1  $CDCl_3$ /CD $_3$ OD):  $\delta$  4.87 (4H, s), 7.34 (4H, d,  $J = 8.4$  Hz), 7.74–7.80 (3H, m), 8.02 (2H, d,  $J = 8.0$  Hz), 8.12 (4H, d,  $J = 8.4$  Hz), 8.15–8.21 (2H, m), 8.30 (2H, d,  $J = 7.6$  Hz), 8.55 (1H, d,  $J = 4.4$  Hz), 8.60–8.69 (3H, m), 9.54 (1H, d,  $J = 5.2$  Hz), 9.65 (1H, d,  $J = 4.8$  Hz), 9.68 (2H, s); High Resolution Q-TOF MS:  $m/z$  887.1414 (calcd for  $C_{49}H_{31}F_3N_2O_6S_2+H$ , 887.1473); Anal. Calcd for  $C_{49}H_{31}F_3N_2O_6S_2$ : C, 68.04; H, 3.61; N, 3.24. Found: C, 67.79; H, 3.37; N, 3.26.

### 4.3. Photophysical properties

**4.3.1. Determination of quantum yields for the generation of singlet oxygen.** The quantum yields for singlet oxygen generation [ $\phi(^1O_2)$ ] of 21,23-dithiaporphyrins, **2–11**, were measured by direct methods in MeOH in a manner identical to the determination of  $\phi(^1O_2)$  for **1**.<sup>13,19</sup> A SPEX 270M spectrometer (Jobin Yvon) equipped with InGaAs photodetector (Electrooptical Systems Inc., U.S.A.) was used for recording singlet oxygen emission spectra. A diode-pumped solid-state laser (Millenia X, Spectra-Physics) at 532 nm was the excitation source. The sample solution, in a quartz cuvette, was placed directly in front of the entrance slit of the spectrometer, and the emission signal was collected at 90° relative to the exciting laser beam. An additional long-pass filter (850LP) was used to attenuate the excitation laser and the fluorescence from the photosensitizer.

**4.3.2. Determination of quantum yields for fluorescence.** The quantum yields for fluorescence ( $\phi_F$ ) of the core-modified porphyrins were measured in MeOH, as described previously,<sup>20</sup> and were compared to the standard Rhodamine 6G ( $\phi_F = 1.0$ ). Steady-state fluorescence spectra of the porphyrins were measured with excitation at 532 nm using Fluorolog-3 spectrofluorometer (Jobin Yvon).

**4.3.3. Determination of *n*-octanol/water partition coefficients at pH 7.4.** The *n*-octanol/water partition coefficients were determined at pH 7.4 using the absorbance of the core-modified porphyrins. A 'shake-flask' direct measurement<sup>17</sup> with 3–5 min mixing, followed by a 4 h settling period, was used. Equilibration and measurements were made at 23 °C using a Perkin-Elmer Lambda 12 spectrophotometer. Values are reported as log  $D_{7.4}$ .

### 4.4. Biology

**4.4.1. Cells and culture conditions.** Cells cultured from the rodent mammary adenocarcinoma (R3230AC) were used for these studies. The R3230AC tumors were maintained by transplantation in the abdominal region of 100–120 g Fischer female rats, using the sterile trocar technique described earlier by Hilf et al.<sup>21</sup> R3230AC cells were cultured from tumor homogenates using the method described earlier.<sup>22</sup> All cell lines were maintained in passage culture on 35 mm diameter polystyrene dishes (Becton Dickinson, Franklin Lakes, NJ) in

3.0 mL of minimum essential medium ( $\alpha$ -MEM) supplemented with 10% FBS, 50 units/mL penicillin G, 50  $\mu$ g/mL streptomycin, and 1.0  $\mu$ g/mL Fungizone (complete medium). Only cells from passages 1 to 10 were used for experiments and cells from passages 1 to 4, stored at  $-86^{\circ}\text{C}$ , were used to initiate cultures. Cultures were maintained at  $37^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  humidified atmosphere (Forma Scientific, Marietta, OH). Passage was accomplished by removing the culture medium, adding a 1.0 mL solution containing 0.25% trypsin, and incubating at  $37^{\circ}\text{C}$  for 2–5 min to remove the cells from the surface, followed by seeding new culture dishes with an appropriate number of cells in 3.0 mL  $\alpha$ -MEM. Cell counts were performed using a particle counter (model ZM, Coulter Electronics, Hialeah, FL).

#### 4.4.2. Incubation of cell cultures with dithiaporphyrins.

For experiments designed to determine the amount of intracellular porphyrin after incubation with core-modified porphyrins, R3230AC cells were seeded on 96-well plates as above. Compounds **1–11** were added at appropriate concentrations in complete medium 24 h after cell seeding. Cells were incubated at  $37^{\circ}\text{C}$  in the dark for various periods, the medium was removed, monolayers were washed once with 0.9% NaCl, and 200  $\mu$ L of a 25% solution of Scintigest (100% DMSO was used for compound **9**) was added to solubilize the cells. The intracellular porphyrin content was determined using a fluorescence multi-well plate reader (Molecular Devices, Sunnyvale, CA) set at appropriate excitation and emission wavelengths. Intracellular dye concentration was determined by comparing fluorescence values obtained from solubilized cells with dye standards dissolved in 25% Scintigest (100% DMSO was used for compound **9**). Data are expressed as femtomole porphyrin/cell.

For experiments designed to determine cell viability in the presence of individual core-modified porphyrins **1–11** in the dark or after light exposure, R3230AC cells were seeded on 96-well plates at  $1\text{--}1.5 \times 10^4$  cells/well in complete medium. Cultures were then incubated for 24 h after which appropriate concentrations of **1–10** were added directly to the wells in complete medium.

**4.4.3. Irradiation of cultured cells.** Following incubation of R3230AC cells with porphyrins, the medium was removed, cells were washed once with 0.2 mL of 0.9% NaCl and 0.2 mL of medium minus FBS, and phenol red (clear medium) was added. Plates, with lids removed, were positioned on an orbital shaker (LabLine, Melrose Park, IL) and exposed for various times to broadband visible light (350–750 nm) delivered at  $1.4\text{ mW cm}^{-2}$  from a filtered 750 W halogen source defocused to encompass the whole 96-well plate. The culture plates were gently orbited on the shaker to ensure uniform illumination of all wells on the plate. The clear medium was then removed, 0.2 mL of fresh complete medium was added, and cultures were incubated at  $37^{\circ}\text{C}$  for 24 h in the dark. Cell monolayers were also maintained in the dark undergoing the same medium changes and addition of dyes as those that were irradiated. Cell counts, as above, were performed on irradiated cells, cells maintained in the dark or cells exposed to

neither porphyrins nor light (control cells). Cell viability, obtained for experimental samples, is expressed as the percent of control cell counts.

**4.4.4. Statistical analyses.** All statistical analyses were performed using Student's *t*-test for pairwise comparisons. A *P* value of  $<0.05$  was considered significant.

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# Phototoxicity of a core-modified porphyrin and induction of apoptosis

Youngjae You<sup>a,\*</sup>, Scott L. Gibson<sup>b</sup>, and Michael R. Detty<sup>a</sup>

<sup>a</sup>*Department of Chemistry, University at Buffalo, The State University of New York, Buffalo, New York 14260-3000*

<sup>b</sup>*Department of Biochemistry and Biophysics, University of Rochester Medical Center, 601 Elmwood Avenue, Box 607, Rochester, New York 14642*

## Abstract

A core-modified porphyrin, 5-phenyl-10,15-bis(carboxylatomethoxyphenyl)-20-(2-thienyl)-21,23-dithiaporphyrin (IY69) was studied in vitro for photodynamic activity under a variety of experimental protocols. Variables included the cell line (the rodent mammary tumor cell line R3230AC or the human breast cancer cell line MCF-7), light fluence, time of exposure of the cell cultures to IY69, and the time post-irradiation for cell counting. The length of time cell cultures were exposed to IY69 impacted cellular accumulation and cellular localization, phototoxicity, and the apparent mode of cell death – apoptosis vs. necrosis.

**Keywords:** core-modified porphyrin; breast cancer; photodynamic therapy; apoptosis

\* Corresponding author. Tel.: +1 716 645 6800 ext. 2197; fax: +1 716 645 6963.

*E-mail address:* yjyou@nsm.buffalo.edu (Y. You).

## 1. Introduction

Photodynamic therapy (PDT) is a promising strategy for the treatment of cancer [1-4]. PDT has regulatory approval from numerous agencies in the US, Canada, Japan, Great Britain, and Europe for treating a variety of human malignancies [1-5]. PDT requires three components: a photosensitizer, oxygen, and light. PDT is made more effective by increasing the selectivity of the photosensitizer for targeted tissues and by the selective delivery of light via fiber optics. Singlet oxygen is the primary phototoxic specie generated by most photosensitizers upon irradiation and the damage induced by singlet oxygen results first in injury to cellular function and structure, and ultimately in cell death and regression of lesions [6]. One major focus of PDT research has been directed towards synthesizing more effective photosensitizers [7]. The criteria for an effective photosensitizer are that it (1) is chemically pure and of known composition, (2) has minimal dark toxicity, (3) has preferential uptake and/or retention by tissues of interest, (4) has rapid excretion leading to low systemic toxicity, (5) has high quantum yield for the generation of singlet oxygen ( $^1\text{O}_2$ ), and (6) has a strong absorbance with a high extinction coefficient in the 600-800-nm range where penetration of light into tissue is maximal [8].

We have prepared new photosensitizers, the core-modified porphyrins, that possess physical and photophysical properties that are desirable for PDT [9-11]. These molecules are prepared via flexible synthetic schemes that allow the photosensitizers to be tailored for specific applications. The substitution of the heavy atoms S or Se for the core nitrogen atoms of natural porphyrins provides unique physicochemical properties. The core-modified porphyrins do not bind metals due to larger atomic sizes of S or Se in the 21- and 23-positions [9]. More importantly, a red-shift in light absorption from 630 to 690 nm occurs. These compounds have low dark toxicity as evidenced in earlier experiments where skin photosensitivity was minimal in a murine model, following exposure to a disulfonated core-modified porphyrin and light [9]. Our previous studies of core-modified porphyrins *in vitro* demonstrated that 5-phenyl-10,15-bis(4-

carboxylatomethoxyphenyl)-20-(2-thienyl)-21,23-dithiaporphyrin (**IY69**), showed the greatest potential as a photosensitizer [12].

Photodynamic responses are highly dynamic processes and experimental factors impact biological results both in vitro and in vivo. We report our initial biological studies in vitro for PDT with **IY69** varying several experimental variables: cell line, concentration of the photosensitizer, fluence of light, and time. To examine the pathway of cell death, we determined the induction of apoptosis after PDT with **IY69**. We find that the phototoxicity and the extent of apoptotic cell death using **IY69** as a photosensitizer are significantly affected by the time for which cultured cells are incubated with **IY69** and the concentration of **IY69**.

## **2. Materials and methods**

### *2.1. Chemicals and reagents*

The detailed synthesis of compound **IY69** appears in an earlier report [12]. Stock solutions of compound **IY69** were prepared in DMSO at  $2 \times 10^{-3}$  M. Serial dilutions were made in sterile doubly distilled water for addition to culture medium. Solvents and reagents were used as received from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted. The Cell Death Detection ELISA<sup>PLUS</sup> assay kit for the detection of apoptosis was purchased from Roche Diagnostics GmbH (Penzberg, Germany). Cell culture medium and antibiotics were purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA).

### *2.2. Cells and culture conditions*

The cell lines used for these studies were the R3230AC rat mammary adenocarcinoma cell line and the human breast tumor cell line MCF-7. The cells were maintained in passage culture on 60 mm diameter polystyrene dishes (Becton Dickinson, Franklin Lakes, NJ) in 3.0 mL of minimum essential medium,  $\alpha$ -MEM for the R3230AC cells and Dulbecco's modified essential medium (D-MEM) for the MCF-7 cells,



supplemented with 10% FBS, 50 units/mL penicillin G, 50 µg/mL streptomycin, and 1.0 µg/mL Fungizone (complete medium). Only cells from passages 1-10 were used for experiments and cells from passages 1-4, stored at -86 °C, were used to initiate cultures. Cultures were maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere (Forma Scientific, Marietta, OH). Passage was accomplished by removing the culture medium, adding a 1.0 mL solution containing 0.25% trypsin, incubating at 37 °C for 2-5 min to remove the cells from the surface followed by seeding new culture dishes with an appropriate number of cells in 3.0 mL of medium. Cell counts were performed using a particle counter (model ZM, Coulter Electronics, Hialeah, FL).

### *2.3. Determination of intracellular accumulation*

The amount of intracellular dye was determined using the fluorescence emission of compound **IY69** at 720 nm. Cultured R3230AC or MCF-7 cells were seeded on 96 well plates in the appropriate medium at cell densities ranging from 2 to 3×10<sup>4</sup> cells/well and incubated for 24 h. Compound **IY69** was added at 5×10<sup>-6</sup> M and incubated with the cell monolayer for selected periods from 1 to 24 h. The medium was removed, cells were washed twice with 0.9% NaCl and 0.2mL of 25% Scintigest™ were added to the monolayer and incubated for 1 h at 37 °C. The fluorescence in the cell digests was determined using the multi-well fluorescence plate reader. Excitation at 440 nm produced a peak emission at 720 nm which was used to determine intracellular concentration of **IY69**. Cell numbers were determined as above and the intracellular accumulation of **IY69** was calculated from a fluorescence standard curve generated from known concentrations of **IY69** dissolved in Scintigest™. The cellular concentrations of **IY69** are expressed as femtomole/cell.

### *2.4. Determination of dark- and phototoxicity (MTT assay)*

Cytotoxicity (dark- or phototoxicity) was determined by MTT assay [13]. Briefly, cells (1.0-1.2 x 10<sup>4</sup> cells for R3230AC; 5-7 x 10<sup>3</sup> cells for MCF-7 cells) in 190 µL complete medium were plated in 96 well plates. Following incubation of cultured

R32320AC or MCF-7 cells with various concentrations of **IY69** for 24 h, the medium was removed, cells were washed twice with 0.2 mL of 0.9% NaCl, and 0.2 mL/well of medium minus FBS and phenol red (clear medium) was added. The plates, with lids removed, were positioned on a orbital shaker (LabLine, Melrose Park, IL) and exposed for various time (less than 1 hr) to broadband visible light (350-750 nm) delivered at 1.4 mW cm<sup>-2</sup> from a filtered 750 W halogen source defocused to encompass the whole 96 well plate. The culture plates were gently orbited on the shaker in order to ensure uniform illumination of all of the wells on the plate. The clear medium was then removed, 0.2 mL of fresh complete medium was added and cultures were incubated at 37 °C for various times up to 24 h in the dark. To determine dark-toxicity, cell monolayers were also maintained in the dark undergoing the same medium changes and dye additions as those that were irradiated. Cytotoxicity, either dark- or photo-toxicity, was determined using the MTT assay 24 h after the irradiation and expressed as the percent of controls, cells exposed to neither porphyrins nor light.

### *2.5. Cell counting with a particle counter*

To determine cell number with or without irradiation, cells were detached with 1x trypsin-EDTA and an aliquot of 0.2 ml trypsin solution was added to each well. The plates were incubated at 37°C until the cells lifted from the surface (approximately 5 min). Cell counts were performed using a particle counter (Model ZM, Coulter Electronics, Hialeah, FL, USA).

### *2.6. Photodynamic treatment and determination of apoptotic cell death and number of cells*

Apoptotic cell death was determined by measuring the DNA fragments (nucleosomes) using the Cell Death Detection ELISA<sup>PLUS</sup> kit and the details followed the instructional manual (Roche Diagnostics, Cat. No. 11 920 685 001) [14]. Cell numbers were determined with the Coulter counter as described above. R3230AC cells were plated on 96 multi-well tissue culture plates at a concentration of  $5 \times 10^3$  cells per well in

190  $\mu$ L complete medium and incubated for 24 h. Various concentrations of **IY69**,  $5 \times 10^{-8}$  to  $4 \times 10^{-6}$  M, were added to the monolayers and incubated for 4 or 24 h at 37 °C in the dark. Following the incubation periods the medium containing **IY69** was removed, 200  $\mu$ L of clear medium was added to the wells and cells were irradiated as described in the section for measurement of phototoxicity. Clear medium was replaced with 200  $\mu$ L complete medium, the cells were incubated additionally for 2 h. Cells were counted with a particle counter to determine the number of live cells. To determine apoptotic cell death, cells were lysed and placed into a streptavidin-coated 96 well plate. By adding a mixture of anti-histone-biotin and anti-DNA-peroxidase, mono- and oligonucleosomes were captured forming sandwich immunocomplexes. Absorbances at 405 and 490 nm were measured after adding ABTS [2,2'-azinodi-(3-ethylbenzthiazoline sulfonate)] substrate. The difference of absorbances (ABS) per  $10^4$  cells was calculated for the unit of nucleosomal production.

$$\text{Unit of nucleosomal production} = \text{ABS [405 - 490 nm]} / 10^4 \text{ cells}$$

## 2.7. Statistical Analysis

Statistical analyses were performed using the Student's t-test. For all tests, P-value of less than 0.05 was considered to be a statistically significant difference.

## 3. Results and discussion

### 3.1. Time dependent intracellular accumulation into cultured R3230AC or MCF-7 cells

Cultured R3230AC cells were exposed to  $5 \times 10^{-6}$  M **IY69** for various times. Such a high concentration was employed due to the low intrinsic fluorescence of **IY69**. The intracellular concentration of **IY69** reached a plateau after 6-7 h (Fig. 1). Longer incubation times gave no significant increase in cellular accumulation.

### 3.2. Dark- and phototoxicity towards cultured R3230AC or MCF-7 Cells

No dark toxicity was observed upon incubation of either R3230AC or MCF-7 cells with up to  $1 \times 10^{-6}$  M **IY69** for 24 h as shown in Figure 2. However, both cell lines showed significant phototoxicity upon irradiation following exposure to **IY69** at concentrations between  $1 \times 10^{-7}$  M and  $1 \times 10^{-6}$  M. Comparable concentrations of **IY69** were more phototoxic toward R3230AC cells than toward MCF-7 cells, which is consistent with the lower intracellular accumulation of **IY69** in the MCF-7 cells (data not shown). However, we cannot exclude other possibilities including a lower level of caspase-3 in MCF-7 cells [15] since at lower concentrations of **IY69** ( $< 3 \times 10^{-7}$  M), apoptosis appears to be an important mechanism of cell death as described below in Section 3.6. It has been demonstrated that MCF-7c3 cells, which express more caspase-3 than MCF-7v cells, are more sensitive to apoptotic cell death with the photosensitizer Pc4 than MCF-7v cells although the overall efficacy either in vitro or in vivo is not related to caspase-3 levels in MCF-7 cells [16, 17].

### *3.3. Porphyrin and light dose dependency of phototoxicity in cultured R3230AC cells*

The data displayed in Figure 3 demonstrate that phototoxicity towards cultured R3230AC was both porphyrin and light-dose dependent. It appears that the minimum doses required to achieve 40% phototoxicity are  $1 \times 10^{-7}$  M **IY69** combined with at least  $3.75 \text{ J/cm}^2$  of broad band white light delivered at  $1.4 \text{ mW/cm}^2$ . Although **IY69** does not absorb the whole range of broad-band light, its absorption is expected to be proportional to the total fluence of the light. The data also show that a relationship between porphyrin dose and total fluence exists. For example, increasing the porphyrin dose allows for a concomitant decrease in the total amount of light delivered to achieve the same level of phototoxicity.

### *3.4. Effects of time after irradiation on phototoxicity towards cultured R3230AC cells*

The data displayed in Figure 4 represent the phototoxicity toward R3230AC cells at various times after the irradiation with **IY69**. Cell counts were determined at different

times, 1, 6, 12, and 24 h after irradiation and data are reported as the ratio of the number of live cells in treated samples to the number of live cells in controls. The data demonstrate that the cell number was significantly decreased by 6 h after irradiation, compared to the number of control cells, for compound **IY69** at  $1 \times 10^{-7}$  M or greater concentration. **IY69** concentrations higher than  $2 \times 10^{-7}$  M plus light continued to elicit a significantly greater decrease in cell viability over the whole 24 h period. Concentrations less than  $5 \times 10^{-8}$  M **IY69** imposed little or no significant phototoxicity until 24 h after irradiation.

Interestingly enough, at  $1 \times 10^{-7}$  M **IY69** cell number was constant up to 12 h after irradiation. To keep the cell number constant up to 12 h, there were two possibilities. First, the initial cells did not proliferate without cell death, i.e. cytostatic effect. Secondly, some cells died but the other cells doubled quickly to compensate the number of dead cells. Scott et al. reported cytostatic effect of photodynamic therapy of 0.05 mM  $\alpha$ -ALA with  $30 \text{ mJ/cm}^{-2}$  irradiation [18]. It is not clear which is the case in this experiment. However, cell number slightly increased at 24 h although overall phototoxicity at 24 h was 54% compared to the control, which means cells regain their proliferate capacity. It will be interesting to observe the long term effects on phototoxicity and more detailed mechanistic study which will elucidate dynamic cellular responses to low dose photodynamic treatment with **IY69**.

### *3.5. Effect of incubation time of cells with **IY69** before irradiation on phototoxicity*

Phototoxicity was determined for cultured R3230AC cells incubated with compound **IY69** for various times prior to irradiation. The cells were exposed to **IY69** for 1, 3, 5, 7, 9, 12, 18, or 24 h, then, irradiated with  $5 \text{ J/cm}^{-2}$  of broad-band light. Phototoxicity was determined 24 h after the irradiation. The data displayed in Figure 5 show that the longer cells are incubated with compound **IY69**, the more effective the subsequent light treatment becomes. At higher concentrations, e.g. ( $1 \times 10^{-6}$  M), significant levels of cytotoxicity can be reached at much shorter incubation intervals, within 6 h with incubation time-dependent manner. If we assume that the kinetics of

uptake at these concentrations, 1, 2, or  $10 \times 10^{-7}$  M, is similar to that at  $5 \times 10^{-6}$  M (Fig. 1), the time-dependent phototoxicity of **IY69** up to 6 h is consistent with the time-dependent concentration of **IY69** in cells.

Incubation time-dependency of phototoxicity was also absorbed after 6 h in cells treated with 2 or  $1 \times 10^{-7}$  M **IY69**. The difference of phototoxicity at 6 and 24 h with  $1 \times 10^{-7}$  and  $2 \times 10^{-7}$  M can not be explained by the difference in concentration because the amounts of **IY69** at 6 and 24 h are expected to be similar. In our fluorescence microscopic study, the distributions of fluorescence from **IY69** in cells at 4 and 24 h after addition were different: most of fluorescence seemed evenly distributed throughout cells after 4 h (data not shown). On the other hand, localized bright spots of the fluorescence were absorbed in cells at 24 h after the addition of **IY69** without staining nuclei [12]. The sites of localization of photosensitizers are the sites of initial photodamage by singlet oxygen generated by photodynamic therapy: it has been generally believed that life time of singlet oxygen is short,  $0.03 \sim 0.18 \mu\text{s}$  *in vivo*, thus diffusion distance of singlet oxygen is very limited [19]. Thus, we presume that the localization of **IY69** in specific sites inside cells with longer incubation make the photosensitizer more efficient in expressing phototoxicity.

### *3.6. Effect of incubation time of cells with **IY69** on induction of apoptotic cell death*

We determined the degree of photo-induced apoptosis after 4- or 24-h incubation of cultured R3230AC cells with various concentrations of **IY69** as well as the number of cells in parallel for phototoxicity. Apoptotic cell death was measured using the Cell Death Detection ELISA<sup>PLUS</sup> kit provided by Roche diagnostics. This kit determines the presence of mono- and oligonucleosomes in the cytosol of cell lysates as one representative indication of the DNA degradation following the induction of apoptosis [20, 21]. The data displayed in Figure 6 reveal the importance of both the concentration of **IY69** and the time of exposure of cultured R3230AC cells to **IY69** on both phototoxicity and induction of apoptosis.

The level of phototoxicity was dependent on the concentration of **IY69** for cells exposed for either 4 h or for 24 h to the photosensitizer (Figure 6a). For any given concentration, 24-h exposure to the photosensitizer gave increased phototoxicity relative to a 4-h exposure. As an example, exposure to  $5 \times 10^{-7}$  M **IY69** and light gave nearly 100% phototoxicity while a 4-h exposure gave around 40% phototoxicity.

The production of mono- and oligonucleosomes due to the induction of apoptosis was affected by the concentration of **IY69** (Fig. 6b). However, the effect did not solely depend on the concentration of **IY69**. There were optimal concentrations for the maximal production of nucleosomes for both 4 and 24 h-exposure conditions, 0.3 and 2.1 units (ABS[405-490]/ $1 \times 10^4$  cells) at  $1 \times 10^{-6}$  and  $2 \times 10^{-7}$  M **IY69**, respectively. At higher concentrations above the optimal conditions, the production of nucleosomes decreased (Fig. 6b). The production of nucleosomes reached minimal points, 0.53 and 0.094 units at high concentrations,  $5 \times 10^{-7}$  and  $4 \times 10^{-6}$  M **IY69** for 4 and 24-exposures. The concentration of photosensitizers in cells was reported as one of the main factors influencing pathway of cell death after PDT [22-26]. It was consistent in PDT with **IY69** that the mode of cell death shifted pathway from pro-apoptotic to pro-necrotic as concentration of a photosensitizer increased.

The other interesting result was the effect of exposure time of **IY69** to the cells on induction of apoptosis. Overall, PDT with 24 h-exposure of **IY69** produced more nucleosomes than that with 4 h-exposure. At the optimal conditions, longer exposure of **IY69** to R3230AC cells at lower concentration,  $2 \times 10^{-7}$  M for 24 h, produced the more nucleosomes than shorter exposure at higher concentration,  $1 \times 10^{-6}$  M for 4 h. The production of nucleosomes at  $2 \times 10^{-7}$  M with 24 h-exposure was seven times the amount of nucleosomes at  $1 \times 10^{-6}$  M with 4 h-exposure: 2.1 vs. 0.3 units. Considering this result with the intracellular localization 24 h after addition of **IY69** [12], it is likely that the sites of localization of **IY69** with 24 h-exposure were important organelles for apoptosis. This hypothesis is supported by previous reports where the sites of localization of photosensitizers in cells were primary photodamage sites, consequently important in determining cell death pathway in PDT: apoptosis or necrosis [6, 22, 27-31].

#### **4. Conclusion**

The new photosensitizer, **IY69**, was phototoxic towards both cultured R3230AC rat mammary tumor cells and MCF-7 human breast cancer cells. The phototoxicity toward R3230AC cells exposed to **IY69** and light was dependent on various experimental conditions such as concentration of the **IY69**, total fluence of light, the length of time post irradiation, and exposure time of **IY69** to cultured R3230AC cells. Especially, the time of exposure of **IY69** to R3230AC cells was a main factor for cellular concentration, localization, and cell death mechanism after irradiation. We are thus under process to find molecular mechanistic and pharmacokinetic profiles of photodamage and localization in PDT with **IY69**.

#### **Acknowledgements**

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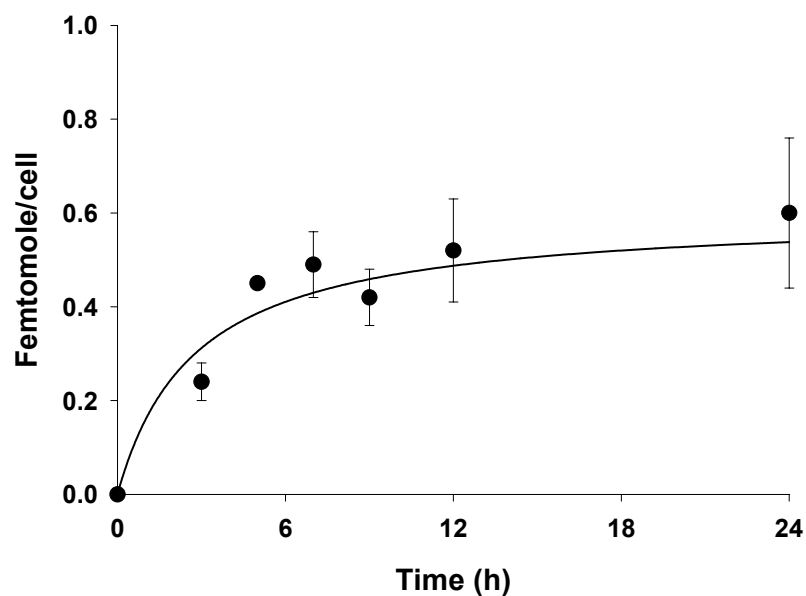
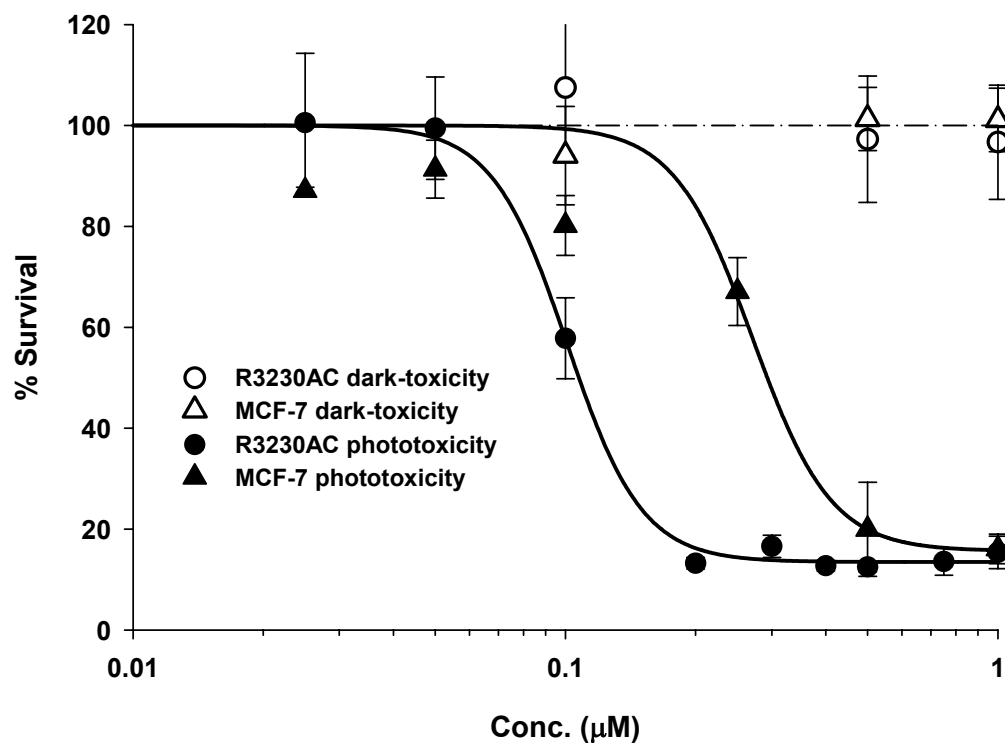


Fig. 1. Time course of intracellular accumulation of **IY69**. Cultured R3230AC cells were incubated with  $1 \times 10^{-6}$  M of **IY69** for various times and the intracellular concentration of **IY69** was determined from its fluorescence. Cell culture conditions, fluorescence determination and calculation of intracellular dye content are detailed in the Methods section. Each data point represents the intracellular concentration of **IY69**, expressed as femtomole/cell, determined in 3 separate experiments performed in duplicate, error bars are the SEM.



1) plate cells	2) add IY69	3) irradiate	4) MTT
0 h	24 h	48 h	72 h

Fig. 2. Dark and phototoxicity of compound **IY69** towards cultured R3230AC cells (closed symbols) and MCF-7 cells (open symbols). Cell culture and irradiation conditions are detailed in the Methods section. Each data point represents the mean of at least 3 separate experiments performed in duplicate, bars are the SEM. Data are expressed as the percent of viable cells compared to control cells (cells not exposed to compound **IY69** or light).

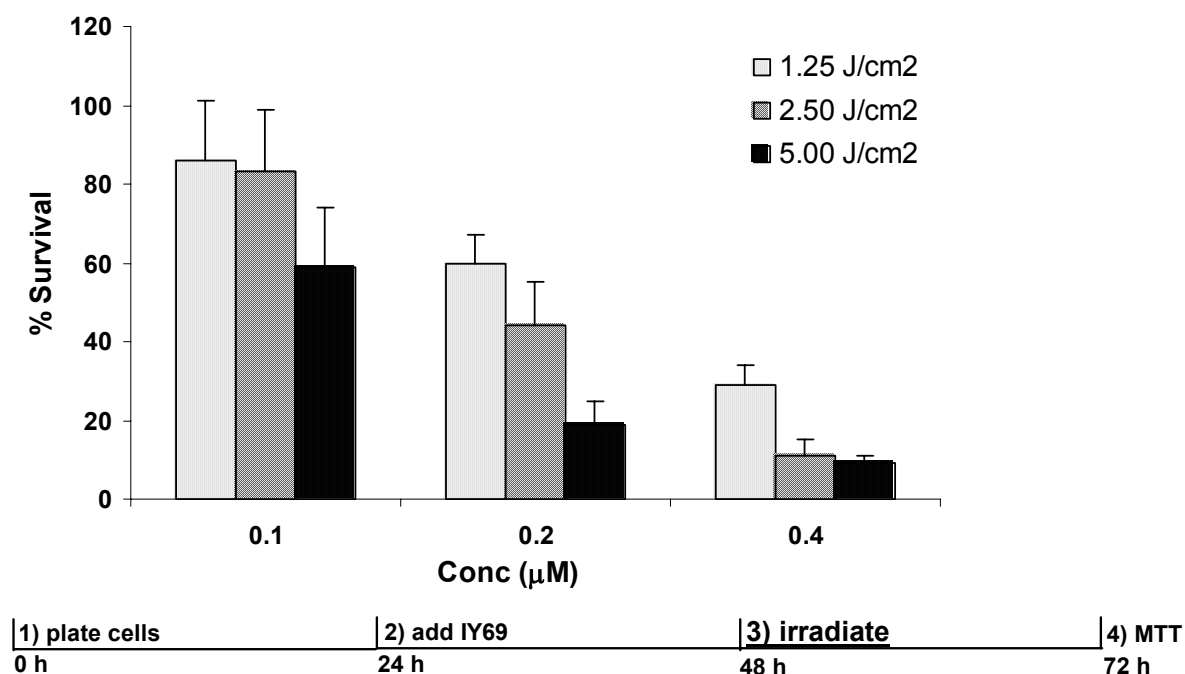


Fig. 3. Porphyrin and light dose related phototoxicity towards cultured R3230AC cells. Cell culture, irradiation conditions and viability determinations using MTT are detailed in the Methods section. Each data point represents the mean of at least 3 separate experiments performed in duplicate, bars are the SEM. Data are expressed as the percent of toxicity compared to control cells (cells not exposed to compound **IY69** or light).

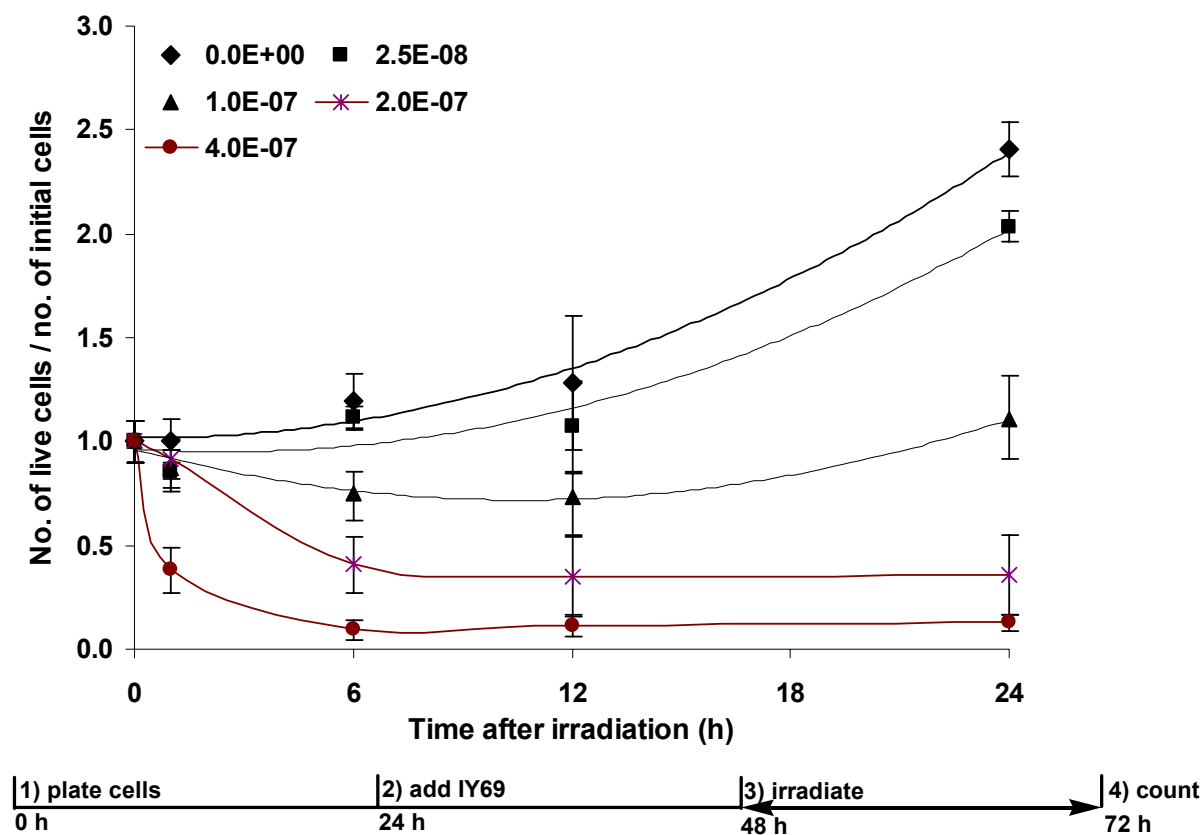


Fig. 4. Time course of R3230AC phototoxicity after irradiation of monolayers exposed to compound **IY69**. Cell culture and irradiation conditions are detailed in the Methods section. Each data point represents the mean of at least 2 separate experiments performed in triplicate, bars are the SEM. Data are expressed as ratios of cell numbers counted at various times after irradiation with compound **IY69** at concentrations ranging from 0 to  $4 \times 10^{-7}$  M.

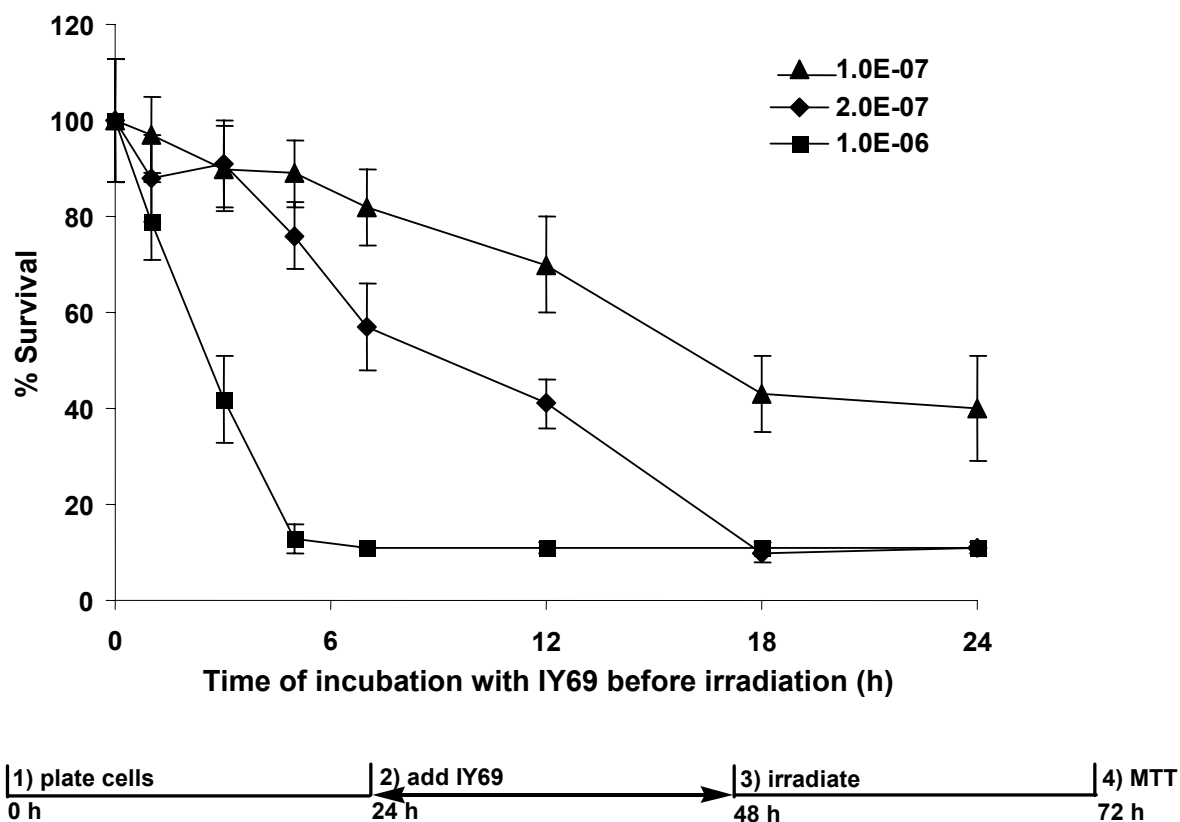
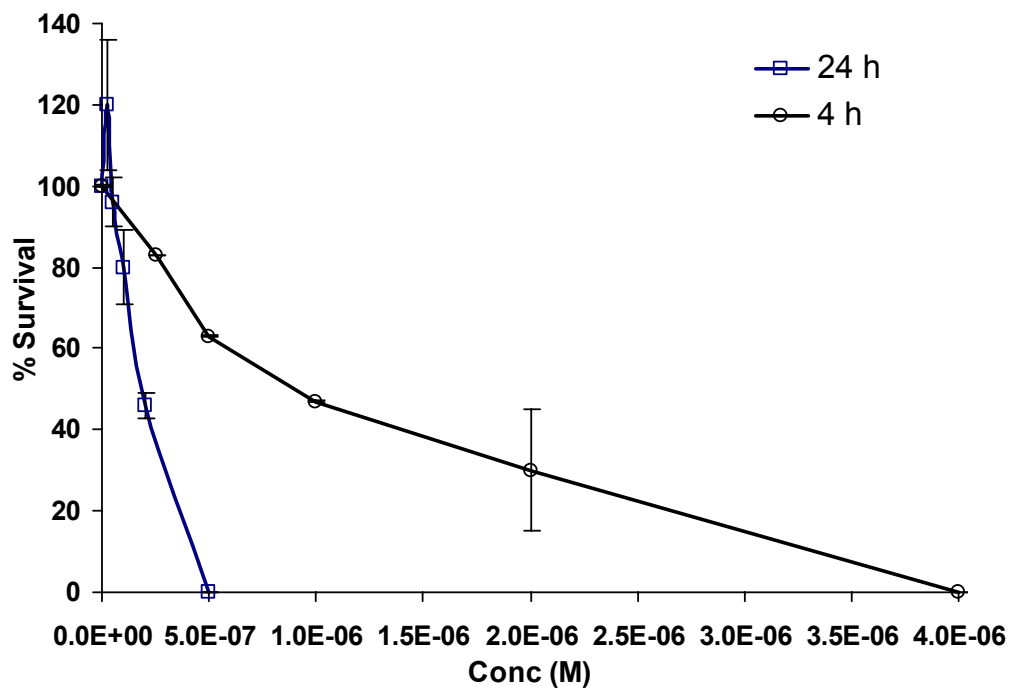
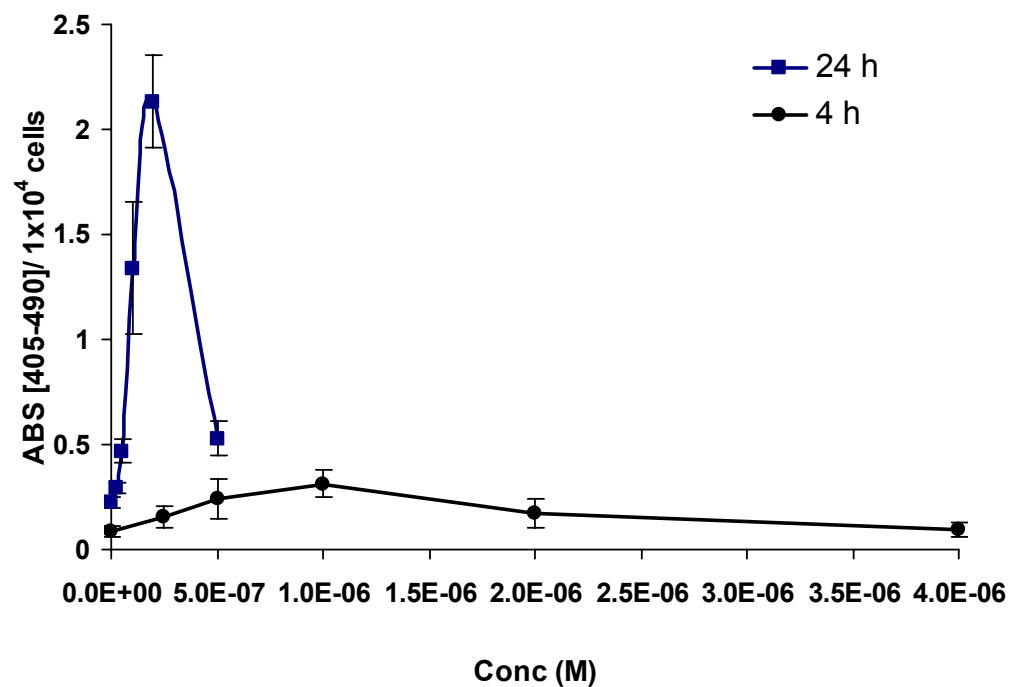


Fig. 5. Effect of time of exposure of cultured R3230AC cells to compound **IY69** prior to irradiation on phototoxicity. Cell culture, irradiation conditions and cytotoxicity determinations using MTT are detailed in the Methods section. Each data point represents the mean of at least 3 separate experiments performed in duplicate, bars are the SEM. Data are expressed as the percent of control cell cytotoxicity (cells not exposed to compound **IY69** or light).





a)



b)

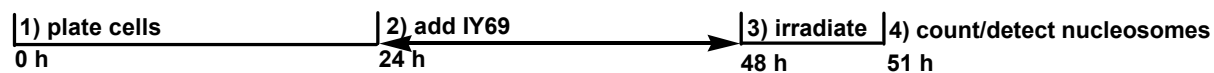


Fig. 6. Determination of cell number and apoptosis. Cell culture and irradiation conditions are detailed in the Methods section. Each data point represents the mean of at least 3 separate experiments performed in duplicate, bars are the SEM.

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### SAR study of new core-modified porphyrins as photosensitizers for photodynamic cancer therapy

**Youngjae You**<sup>1</sup>, Scott L Gibson<sup>2</sup>, Russell Hilf<sup>2</sup>, and Michael R. Detty<sup>1</sup>. (1) Department of Chemistry, The State University of New York, Buffalo, 630 Natural Sciences Complex, Buffalo, NY 14260, Fax: 716-645-6963, yjyou@nsm.buffalo.edu, (2) Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry

To observe the SAR relationships of 21,23-dithiaporphyrins with physicochemical and biological properties as photosensitizers, derivatives of 5,20-diphenyl-10,15-bis(4-carboxylatomethoxy)phenyl-21,23-dithiaporphyrin were prepared and their physicochemical and biological properties were evaluated. The structural change was focused on both the steric bulkness of 5- and 20-meso positions and the symmetry of the molecules. Minor impacts were observed in physicochemical properties such as the UV-VIS-near-IR absorption spectra, quantum yields for the generation of singlet oxygen and fluorescence. Some effects were shown in the values of the octanol/water partition coefficient. On the other hand, the modification at the meso positions resulted in significant differences in photosensitizing efficacy towards R3230AC cells: The smaller and unsymmetric porphyrins were better than the others. Of the synthesized compounds, 5-phenyl-20-(2-thienyl)-10,15-bis(4-carboxylatomethoxy-phenyl)-21,23-dithia-porphyrin showed the best phototoxicity: 68% cell kill at 100 nM and irradiation with 5 J/cm<sup>2</sup> of 350-750-nm light.

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## **SAR of new core-modified porphyrins as photosensitizers for photodynamic cancer therapy: Electronic effects and importance of aggregation in biological activity**

### **AEI 52**

**Youngjae You**, Michael R. Detty, Scott L. Gibson, and Russell Hilf.

Derivatives of 5,20-diphenyl-10,15-bis(4-carboxylatomethoxy)phenyl-21,23-dithiaporphyrin were prepared and their physicochemical and biological properties were tested to reveal the SAR relationships of 21,23-dithiaporphyrins with physicochemical and biological properties as photosensitizers. The structural modifications were focused on the diversity in steric and electronic properties of 5- and 20-meso aromatic substituents. Physicochemical properties were not affected much by the steric or electronic variations in meso aromatic rings. However, porphyrin bearing dimethylamino or hydroxyl groups displayed interesting results. The dimethylamino porphyrin manifested red-shifts in Q bands and fluorescence with increased quantum yield of fluorescence and decreased production of singlet oxygen. In stark contrast to the dimethylamino compound, dihydroxy porphyrin lost of both singlet oxygen and fluorescence generation properties in MeOH. Biological activities were very sensitive to steric bulk but somewhat tolerated to electronic effects. Aggregation states of porphyrin seemed to be enormously important in biological properties, which was exemplified in the difference between dimethylamino and dihydroxy porphyrins.

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## STRUCTURE-ACTIVITY RELATIONSHIP OF 21,23-CORE-MODIFIED PORPHYRINS FOR PHOTODYNAMIC THERAPY OF CANCER

**Youngjae You, Scott L. Gibson, Rossell Hilf, and Michael R. Detty**

Department of Chemistry, State University of New York at Buffalo, Buffalo, NY; Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, NY

E-mail: yjyou@buffalo.edu

Photodynamic therapy (PDT) is a developing cancer treatment regimen whose efficacy is dependent on selective accumulation of a photosensitizing agent in malignant tissue and the ability to target lesions with visible light at 600-800 nm. Successful PDT requires three components: light, photosensitizer, and molecular oxygen. One major component of PDT, the photosensitizer, has attracted attention mainly due to the variety of chemical structures that can be used and the ability to manipulate these structures to increase therapeutic efficacy. However, the photosensitizers that are available have deficiencies such as lack of selectivity for malignancies, low light absorption at wavelengths >600 nm and chronic skin phototosensitivity.

We developed core-modified porphyrins to overcome some of these shortcomings. We focused on modifications to the core-modified porphyrins that would alter the bulk of the compounds and/or the hydrogen bonding capacity of the substituents at the mesoposition on the phenyl rings. The dithiaporphyrins we synthesized were evaluated for photophysical and biological properties using cultured rat (R3230AC) and human (MCF-7) breast cancer cells. These studies included intracellular localization of porphyrins, time-dependent uptake, phototoxicity and determinations of the mode of cell death.

The photophysical properties of the dithiaporphyrins studied were highly suitable for an ideal photosensitizer: light absorption at ~ 700 nm, high quantum yields of singlet oxygen (0.6-0.8). The most effective compounds were phototoxic towards cultured cells with IC<sub>50</sub> at 50–200 nM and no dark toxicity. The less bulky and unsymmetric compounds were more effective as photosensitizers, in vitro, than bulkier symmetrical structures. In addition, the steric effects were more important than electronic properties of the substituents. 5-Phenyl-20-(2-thienyl)-10,15-bis-(4-carboxylatomethoxyphenyl)-21,23-dithiaporphyrin (3), was most potent, showed specific intracellular localization patterns and induced apoptotic cell death. In in vivo pharmacokinetic studies, 3 was rapidly eliminated from the blood of rats.

These results, taken together, demonstrate that the dithia-core-modified porphyrin 3 has the photophysical properties necessary for an ideal photosensitizer aspect and that it is a very effective photosensitizer towards tumor cells in vitro which under specific conditions induces apoptosis.

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